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DESCRIPTION

NOVEL NITRILE HYDRATASE

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TECHNICAL FIELD

The present invention relates to a novel nitrile hydratase, as well as to a gene encoding the enzyme, to a plasmid containing the gene, to a transformant strain as transformed with the plasmid, to a method for producing a nitrile hydratase using the transformant strain, and to a method for processing a nitrile compound with a culture obtained from cultivating the transformant strain, with the cultivated cells or with a product obtained by processing the cultivated cells, to produce a corresponding amide compound.

Further, the invention relates to a process for modifying the properties of an enzyme having the activity of nitrile hydratase. Furthermore, the invention relates to an enzyme with modified properties, as well as to a gene encoding the enzyme, to a plasmid containing the gene, to a transformant strain transformed with the plasmid, to a method for producing a nitrile hydratase using the transformant strain, and to a method for processing a nitrile compound with a culture obtained from cultivating

the transformant strain, with the cultivated cells or with a product obtained by processing the cultivated cells, to produce a corresponding amide compound. The invention is useful in the field of material production using a  
5 biological catalyst.

#### BACKGROUND ART

A nitrile hydratase has been discovered which is an  
10 enzyme having the nitrile-hydrating activity to convert a nitrile group of various compounds to an amide group by hydration, and a number of microorganism strains producing the above-mentioned enzyme have been introduced. In order to produce an amide compound from a nitrile compound using a  
15 nitrile hydratase on an industrial scale, it is important to reduce the proportion of the production costs for this enzyme in the total production costs for producing the amide compound. More specifically, it is necessary to increase the content of the enzyme in a unit weight of the  
20 preparation obtained from the enzyme production. Thus, attempts have been being made to clone the gene of the enzyme for the purpose of expressing a large amount of the enzyme through genetic engineering means using the gene of the enzyme.

25 As the microorganisms having the activity of nitrile

hydratase, *Rhodococcus rhodochrous* strain J1 (deposited under the Budapest Treaty with the International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki-ken, Japan, under the deposit number FERM BP-1478) and *Pseudonocardia thermophila* (this strain is in storage in the Japan collection of Microorganisms at RIKEN BioResource Center, 2-1 Hirosawa, Wako-shi, Saitama-ken 351-0198, Japan, under the accession number JCM3095 and may be freely distributed to anyone upon request. Also, it is deposited under the Budapest Treaty with the International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology, under the deposit number FERM BP-7379) are known (see Patent Documents 1 and 3).

In addition, a nitrile hydratase has been isolated from these strains, and it has been confirmed that this enzyme consists of two types of polypeptides generally referred to as an  $\alpha$ -subunit and a  $\beta$ -subunit. Further, the gene of the nitrile hydratase has been isolated from these strains, and the amino acid sequence and the base sequence for the enzyme have been identified. Also, the plasmid which can express the nitrile hydratase in a transformant and the strain transformed with this plasmid (for example, TG1/pNHJ10H and MT-10822: under the Budapest Treaty, the former is deposited

with the International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology under the deposit number FERM BP-2777, and the latter is deposited with the International Patent Organism Depositary at the National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki-ken, Japan, under the deposit number FERM BP-5785, as of February 7, 1996) have been prepared. Additionally, it has been made possible to produce a nitrile hydratase by means of these strains, and to process a nitrile compound to produce a corresponding amide compound by bringing the strain or the nitrile hydratase obtained therefrom into contact with the nitrile compound (see Patent Documents 2 and 4, and Non-patent Document 1).

Also, attempts have been being made to interpret the stereostructure of a nitrile hydratase, and the interpretation results have been disclosed under PDB ID NOs: 1AHJ, 2AHJ and 1IRE. It is clear now that the enzyme comprises a dimer having the  $\alpha$ -subunit and the  $\beta$ -subunit which are in association as the fundamental structural unit, and the dimers are further associated to form tetramers, octamers or dodecamers (depending on the biological species of origin) in order to express the activity. Further, the region or structure forming the active center has been identified, and it is known that the active center is not at



an exposed position on the external side of the enzyme where direct contact is made with the reaction solvent, but at a position where it looks like being embedded inside the enzyme. Also known is the stereostructure in which a metal atom that is essential for expression of the activity (cobalt atom or iron atom, depending on the biological species of origin) is coordinated to the active center, and it has been disclosed that a cysteine residue in the amino acid sequence that constitutes the region forming the active center undergoes oxidation after transcription, as a phenomenon associated with the coordination of a metal atom. Specifically, a region represented by the sequence  $X_1CXLC_1SC_2X_2X_3X_4X_5$  (wherein, C corresponds to cysteine, X to serine or threonine, L to leucine,  $C_1$  to cysteine sulfinic acid (cysteine sulfinic acid-3-sulfinoalanine), S to serine, and  $C_2$  to cysteine sulfenic acid (cysteine sulfenic acid-S-hydroxy-cysteine); and  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$  and  $X_5$  represent arbitrary amino acid, respectively) in the amino acid sequence of the  $\alpha$ -subunit is known as the region responsible for the coordination of the metal atom to the active center (see Non-patent Documents 2 to 4).

However, with regard to the method of modifying the properties of the nitrile hydratase, without impairing the original activity thereof, such as the enzymatic activity, substrate-specificity,  $V_{max}$ ,  $K_m$ , thermal stability, stability

against the substrate, stability against the product or the like, no invention has been reported which discloses a specific technique therefor. In particular, no attempt has been made regarding the method of modifying the above-

5 mentioned properties by paying attention to the stereostructure of the nitrile hydratase and changing the stereostructure.

Furthermore, it is disclosed in Patent Document 5 that in the case of producing a nitrile hydratase having the enzymatic activity by expressing a gene that codes for nitrile hydratase using a host cell, there exists a protein which is involved in the activation of the enzyme.

Patent Document 1: Japanese Patent Application Laid-Open No. 2-470

15 Patent Document 2: Japanese Patent Application Laid-Open No. 4-211379

Patent Document 3: Japanese Patent Application Laid-Open No. 8-56684

20 Patent Document 4: Japanese Patent Application Laid-Open No. 9-275978

Patent Document 5: Japanese Patent Application Laid-Open No. 11-253168

Non-patent Document 1: Kobayashi M, Nishiyama M, Nagasawa T, Horinouchi S, Beppu T, Yamada H. Cloning, 25 nucleotide sequence and expression in *Escherichia coli* of

two cobalt-containing nitrile hydratase genes from  
*Rhodococcus rhodochrous* J1. Biochim Biophys Acta. 1991 Dec  
2; 1129 (1): 23-33.

Non-patent Document 2: Huang W, Jia J, Cummings J,  
5 Nelson M, Schneider G, Lindqvist Y. Crystal structure of  
nitrile hydratase reveals a novel iron centre in a novel  
fold. Structure. 1997 May 15; 5(5): 691-9.

Non-patent Document 3: Nagashima S, Nakasako M, Dohmae  
N, Tsujimura M, Takio K, Odaka M, Yohda M, Kamiya N, Endo I.  
10 Novel non-heme iron center of nitrile hydratase with a claw  
setting of oxygen atoms. Nat Struct Biol. 1998 May; 5(5):  
347-51.

Non-patent Document 4: Miyanaga, A., Fushinobu, S., Ito,  
K., and Wakagi, T. Crystal structure of cobalt-containing  
15 nitrile hydratase. Biochem. Biochem Biophys Res Commun. 2001  
Nov 16; 288(5): 1169-74.

#### DISCLOSURE OF INVENTION

20 It is an advantage of the invention to provide the  
amino acid sequence of a nitrile hydratase which has a novel  
substitution mutation site that does not substantially  
modify the function, and the base sequence of the gene  
encoding the enzyme. Moreover, it is to provide a plasmid  
25 containing the gene, a transformant strain transformed with

the plasmid, a process for producing a nitrile hydratase using the transformant strain, and a process for processing a nitrile compound with a culture obtained from cultivating the transformant strain, with the cultivated cells or with a product obtained by processing the cultivated cells, to produce a corresponding amide compound.

Further, another advantage of the invention is to provide a specific technique relating to a method comprising modification of one or more properties of nitrile hydratase such as the enzymatic activity, substrate specificity,  $V_{max}$ ,  $K_m$ , thermal stability, stability against the substrate, stability against the product or the like, without impairing the enzyme's original activity. More specifically, it is to provide a method for modifying the properties by introducing into the nitrile hydratase gene a mutation which effects a change in the stereostructure of the nitrile hydratase. It is also to provide a nitrile hydratase obtained according to the method for modification, a gene encoding the nitrile hydratase, a plasmid containing the gene, a transformant strain transformed with the gene or the plasmid, a method for producing the nitrile hydratase using the transformant strain, and a method for processing a nitrile compound with a culture obtained from cultivating the transformant strain, with the cultivated cells or with a product obtained by processing the cultivated cells, to produce a corresponding

amide compound.

Under these circumstances, the inventors succeeded in introducing into the nitrile hydratase gene disclosed in Japanese Patent Application Laid-Open No. 9-275978 (Patent Document 4), a novel substitution mutation site that is not disclosed in the above-mentioned publication and determined the base sequence of the gene after the introduction of the mutation. Furthermore, they produced a plasmid containing the gene and a transformant strain transformed with the plasmid. They have also made extensive studies on preparing the enzyme using the strain or processing a nitrile compound using a culture obtained from cultivating the transformant strain, with the cultivated cells or with a product obtained by processing the cultivated cells, to produce a corresponding amide compound. As a result, the invention was completed.

The  $\alpha$ -subunit of the nitrile hydratase according to the invention is characterized in that it has an amino acid sequence containing mutation in which at least one amino acid of the 36<sup>th</sup>, 71<sup>st</sup>, 148<sup>th</sup> and 204<sup>th</sup> amino acids in the amino acid sequence of the  $\alpha$ -subunit as set forth in SEQ ID NO: 1 in the Sequence Listing is substituted by another amino acid.

The  $\beta$ -subunit of the nitrile hydratase according to the invention is characterized in that it has an amino acid

sequence containing mutation in which at least one amino acid of the 10<sup>th</sup>, 32<sup>nd</sup>, 37<sup>th</sup>, 41<sup>st</sup>, 46<sup>th</sup>, 48<sup>th</sup>, 51<sup>st</sup>, 72<sup>nd</sup>, 118<sup>th</sup>, 127<sup>th</sup>, 146<sup>th</sup>, 160<sup>th</sup>, 186<sup>th</sup> and 217<sup>th</sup> amino acids in the amino acid sequence of the  $\beta$ -subunit as set forth in SEQ ID NO: 2  
5 in the Sequence Listing is substituted by another amino acid.

The nitrile hydratase according to the invention is characterized in that it comprises an  $\alpha$ -subunit and a  $\beta$ -subunit, and at least one of these subunits has the above-mentioned mutation.

10 The gene encoding the  $\alpha$ -subunit of the nitrile hydratase according to the invention is characterized in that it codes for an amino acid sequence having mutation in the  $\alpha$ -subunit. The gene encoding the  $\beta$ -subunit of the nitrile hydratase according to the invention is  
15 characterized in that it codes for an amino acid sequence having mutation in the  $\beta$ -subunit.

The gene encoding the nitrile hydratase according to the invention is characterized in that it comprises the gene encoding the  $\alpha$ -subunit and the gene encoding the  $\beta$ -subunit,  
20 and at least one of these subunits has the above-mentioned mutation.

The plasmid of the invention is characterized in having any one of the above-mentioned genes.

The transformant of the invention is characterized in  
25 that it is obtained by transformation of a host cell using

the plasmid.

The production of the nitrile hydratase of the invention is characterized in comprising a step of recovering the nitrile hydratase from the transformant, a culture obtained from cultivation of the transformants, or the product obtained from the processing of the transformants or the culture.

The method for producing an amide compound according to the invention is characterized in comprising a step of bringing a nitrile compound into contact with the nitrile hydratase in an aqueous medium to convert the nitrile compound to a corresponding amide compound.

Moreover, under these circumstances, taking an example from the nitrile hydratase genes disclosed in Patent Document 2 and Patent Document 4, the inventors specified a region to be subjected to mutation, based on the aspect of novelty, and implemented the method for modifying a nitrile hydratase by applying alteration such as substitution, insertion or deletion at the amino acids in the amino acid sequence which correspond to the amino acid residues forming the region. Specifically, the inventors specified the region to be modified in accordance with the purpose, by carrying out an assiduous interpretation with reference to the stereostructure of the nitrile hydratase as disclosed in the Non-patent Documents 2, 3 and 4 and in PDB ID NOs: 1AHJ,

2AHJ and 1IRE. More specifically, by the interpretation of the stereostructure, they specified the region which forms a cavity through which a substrate passes from the outside of the enzyme toward the active center or a product passes from the active center toward the outside of the enzyme, and the region which forms an associative interface between the  $\alpha$ -subunit and the  $\beta$ -subunit which is involved in the formation of dimers or an interface involved in the association of dimers. The method for applying alteration such as substitution, insertion or deletion into an amino acid sequence is not particularly limited and may be exemplified by the technique of mutagenesis which employs the gene recombination technology.

In addition, the inventors observed how the method for modification changes the properties of nitrile hydratase, by determining the base sequence of the gene after the above-mentioned alteration, producing a plasmid containing the gene and a transformant strain transformed with the gene or the plasmid, and then performing the production of the enzyme using the transformant strain or the processing of a nitrile compound with a culture obtained from cultivating the transformant strain, with the cultivated cells or with a product obtained by processing the cultivated cells, to produce a corresponding amide compound. After a close examination on the alteration at various sites in the amino



acid sequence constituting the nitrile hydratase to be modified, and on various modified enzymes obtained therefrom, the inventors eventually accomplished the invention.

Therefore, the invention relates to, in addition to the  
5 above-described features, the following [1] to [22]:

[1] a method for modifying an enzyme having the nitrile hydratase activity, which comprises changing one or more properties selected from the enzyme activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against  
10 the substrate and stability against the product, by specifying certain amino acid residues and performing substitution, insertion or deletion at one or more sites of the specified amino acid residues according to the following procedure:

15 (a) aligning the amino acid sequence of the enzyme having the nitrile hydratase activity before modification, with the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing and the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing,

20 (b) specifying, based on the results of the alignment, the amino acid residues corresponding to the region extending from the 36<sup>th</sup> threonine to the 48<sup>th</sup> asparagine in the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing, and to the region extending from the 31<sup>st</sup>  
25 lysine to the 51<sup>st</sup> phenylalanine and to the region extending

from the 112<sup>th</sup> lysine to the 127<sup>th</sup> leucine in the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing, and

(c) performing substitution, insertion or deletion at  
5 one or more sites of the specified amino acid residues;

[2] a method for modifying an enzyme having the nitrile hydratase activity, which comprises changing one or more properties selected from the enzyme activity, substrate specificity,  $V_{max}$ ,  $K_m$ , thermal stability, stability against  
10 the substrate and stability against the product, by specifying certain amino acid residues and performing substitution, insertion or deletion at one or more sites of the specified amino acid residues according to the following procedure:

15 (d) aligning the amino acid sequence of the enzyme having the nitrile hydratase activity before modification, with the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing and the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing,

20 (e) specifying, based on the results of the alignment, the amino acid residues corresponding to the 36<sup>th</sup>, 48<sup>th</sup>, 71<sup>st</sup>, 148<sup>th</sup>, 188<sup>th</sup> and 204<sup>th</sup> of the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing, and of the amino acid residues corresponding to the 10<sup>th</sup>, 32<sup>nd</sup>, 33<sup>rd</sup>, 37<sup>th</sup>, 40<sup>th</sup>,  
25 41<sup>st</sup>, 46<sup>th</sup>, 48<sup>th</sup>, 51<sup>st</sup>, 61<sup>st</sup>, 72<sup>nd</sup>, 112<sup>th</sup>, 118<sup>th</sup>, 127<sup>th</sup>, 146<sup>th</sup>,

150<sup>th</sup>, 160<sup>th</sup>, 168<sup>th</sup>, 171<sup>st</sup>, 176<sup>th</sup>, 186<sup>th</sup>, 217<sup>th</sup> and 218<sup>th</sup> of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing, and

(f) performing substitution, insertion or deletion at  
5 one or more sites of the specified amino acid residues;

[3] a method for modifying an enzyme having the nitrile hydratase activity, which comprises changing one or more properties selected from the enzymatic activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against  
10 the substrate and stability against the product, by specifying certain amino acid residues and performing substitution, insertion or deletion at one or more sites of the specified amino acid residues according to the following procedure:

15 (g) inferring the stereostructure of the enzyme having the nitrile hydratase activity before modification by carrying out an alignment based on the nitrile hydratase stereostructure and the amino acid sequence as set forth in PDB (Protein Data Bank) ID NO: 1IRE,

20 (h) specifying, based on the stereostructure inferred, the amino acid residues in the regions corresponding to the 2<sup>nd</sup> helix as counted from the N-terminal in Chain 1IRE: A, and to the 1<sup>st</sup> helix and the 2<sup>nd</sup> helix as counted from the N-terminal, the loop portions inserted in the latter helices  
25 and the 3<sup>rd</sup> helix as counted from the C-terminal in Chain

1IRE: B in the nitrile hydratase stereostructure as set forth in PDB ID NO: 1IRE, and

(i) performing substitution, insertion or deletion at one or more sites of the specified amino acid residues;

5 [4] a method for modifying an enzyme having the nitrile hydratase activity, which comprises changing one or more properties selected from the enzymatic activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against the substrate and stability against the product, by  
10 specifying certain amino acid residues and performing substitution, insertion or deletion at one or more sites of the specified amino acid residues according to the following procedure:

(j) inferring the stereostructure of the enzyme having  
15 the nitrile hydratase activity before modification by carrying out an alignment based on the nitrile hydratase stereostructure and the amino acid sequence as set forth in PDB ID NO: 1IRE,

(k) specifying, based on the stereostructure inferred,  
20 the four amino acid residues such as the amino acid residues which correspond to the 89<sup>th</sup> amino acid residue glutamine and the 165<sup>th</sup> amino acid residue glutamic acid as counted from the N-terminal in Chain A, and the amino acid residues which correspond to the 37<sup>th</sup> amino acid residue phenylalanine and  
25 the 48<sup>th</sup> amino acid leucine as counted from the N-terminal in

Chain B in the nitrile hydratase stereostructure as set forth in PDB ID NO: 1IRE,

(1) specifying the amino acid residues whose side-chain front-end heavy atoms are located within 5Å of radius in the  
5 respective stereostructure having each of the side-chain front-end heavy atoms of the four above-specified amino acid residues as the point center, and

(m) performing substitution, insertion or deletion at one or more of the amino acid residues specified in the  
10 above (1);

[5] a method for modifying an enzyme having the nitrile hydratase activity, which comprises changing one or more properties selected from the enzymatic activity, substrate specificity,  $V_{max}$ ,  $K_m$ , thermal stability, stability against  
15 the substrate and stability against the product, by specifying certain amino acid residues and performing substitution, insertion or deletion at one or more sites of the specified amino acid residues according to the following procedure:

20 (n) inferring the stereostructure of the enzyme having the nitrile hydratase activity before modification by carrying out an alignment based on the nitrile hydratase stereostructure and the amino acid sequence as set forth in PDB ID NO: 1IRE,

25 (o) specifying, based on the inferred stereostructure,

the region which forms a cavity through which a substrate passes from the outside of the enzyme toward the active center, or a product passes from the active center to the outside of the enzyme,

5           (p) specifying, among the amino acid residues constituting the above-specified region, the amino acid residues whose alteration leads to a change in the cavity size and further controls the easiness or difficulty in passing of the substrate/product, and

10           (q) performing substitution, insertion or deletion at one or more of the amino acid residues specified in the above (p);

          [6] a method for modifying an enzyme having the nitrile hydratase activity, which comprises changing one or more  
15 properties selected from the enzymatic activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against the substrate and stability against the product, by specifying certain amino acid residues and performing substitution, insertion or deletion at one or more sites of  
20 the specified amino acid residues according to the following procedure:

          (r) inferring the stereostructure of the enzyme having the nitrile hydratase activity before modification by carrying out an alignment based on the nitrile hydratase  
25 stereostructure and the amino acid sequence as set forth in

PDB ID NO: 1IRE,

(s) specifying, based on the stereostructure inferred, the four amino acid residues such as the amino acid residues which correspond to the 89<sup>th</sup> amino acid glutamine (A89Q) and  
5 the 165<sup>th</sup> amino acid glutamic acid (A165E) as counted from the N-terminal in Chain A, and the amino acid residues which correspond to the 37<sup>th</sup> amino acid phenylalanine (B37F) and the 48<sup>th</sup> amino acid leucine (B48L) as counted from the N-terminal in Chain B in the nitrile hydratase stereostructure  
10 as set forth in PDB ID NO: 1IRE,

(t) specifying the amino acid residues which effect a change in at least one of d1 to d3, when the shortest distance between the heavy atoms of the amino acid residue corresponding to A165E and of the amino acid residue  
15 corresponding to B48L is designated as d1; the shortest distance between the heavy atoms of the amino acid residue corresponding to A89Q and of the amino acid residue corresponding to B48L as d2; and the shortest distance between the heavy atoms of the amino acid residue  
20 corresponding to B37F and of the amino acid residue corresponding to B48L as d3, and

(u) performing substitution, insertion or deletion at one or more sites of the specified amino acid residues;

[7] the method for modification according to [6],  
25 wherein the step of (t) is replaced by the following step

(t'):

(t') specifying the amino acid residues which effect a change in at least one of d1 to d5, when the shortest distance between the heavy atoms of the amino acid residue corresponding to A165E and of the amino acid residue corresponding to B48L is designated as d1; the shortest distance between the heavy atoms of the amino acid residue corresponding to A89Q and of the amino acid residue corresponding to B48L as d2; the shortest distance between the heavy atoms of the amino acid residue corresponding to B37F and of the amino acid residue corresponding to B48L is designated as d3; the shortest distance between the heavy atoms of the amino acid residue corresponding to A165E and of the amino acid residue corresponding to B37F as d4; and the shortest distance between the heavy atoms of the amino acid residue corresponding to A89Q and of the amino acid residue corresponding to B37F as d5;

[8] the method for modification according to any one of [1] to [7], wherein the enzyme having the nitrile hydratase activity before modification comprises the two polypeptides [A] and [B] of the following:

[A] a polypeptide consisting of an amino acid sequence which shows homology of at least 40% with the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing, and



[B] a polypeptide consisting of an amino acid sequence which shows homology of at least 25% with the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing;

5 [9] the method for modification according to [8], wherein the polypeptide of [A] is the polypeptide of the following [C], and the polypeptide of [B] is the polypeptide of the following [D]:

[C] a polypeptide consisting of any amino acid sequence  
10 selected from the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing; the amino acid sequence in which substitution, insertion or deletion has been implemented at one or more sites in the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing; and  
15 the amino acid sequence in which at least one amino acid of the 6<sup>th</sup>, 19<sup>th</sup>, 38<sup>th</sup>, 77<sup>th</sup>, 90<sup>th</sup>, 102<sup>nd</sup>, 106<sup>th</sup>, 126<sup>th</sup>, 130<sup>th</sup>, 142<sup>nd</sup>, 146<sup>th</sup>, 187<sup>th</sup>, 194<sup>th</sup> and 203<sup>rd</sup> amino acids in the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing is substituted by another amino acid, and

20 [D] a polypeptide consisting of any amino acid sequence selected from the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing; the amino acid sequence in which substitution, insertion or deletion has been implemented at one or more sites in the amino acid sequence  
25 as set forth in SEQ ID NO: 99 in the Sequence Listing; and

the amino acid sequence in which at least one amino acid of the 20<sup>th</sup>, 21<sup>st</sup>, 108<sup>th</sup>, 200<sup>th</sup> and 212<sup>th</sup> amino acids in the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing is substituted by another amino acid;

5           [10] the method for modification according to [8], wherein the polypeptide of [A] is the polypeptide of the following [E], and the polypeptide of [B] is the polypeptide of the following [F]:

          [E] a polypeptide consisting of an amino acid sequence  
10 showing homology with the amino acid sequence which is encoded by the open reading frame (ORF) composed of from the 704<sup>th</sup> to 1315<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing, and

          [F] a polypeptide consisting of an amino acid sequence  
15 showing homology with the amino acid sequence which is encoded by the ORF composed of from the 1<sup>st</sup> to 680<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing;

          [11] a method for modifying an enzyme having the  
20 nitrile hydratase activity, which comprises changing one or more properties selected from the enzymatic activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against the substrate and stability against the product, by specifying certain amino acid residues and performing  
25 substitution, insertion or deletion at one or more sites of

the specified amino acid residues according to the following procedure:

(d') aligning the amino acid sequence of the enzyme having the nitrile hydratase activity before modification,  
5 with the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing,

(e') specifying, based on the results of the alignment, the amino acid residues corresponding to the 48<sup>th</sup> and 51<sup>st</sup> in the amino acid sequence as set forth in SEQ ID NO: 99 in the  
10 Sequence Listing, and

(f') performing substitution, insertion or deletion at one or more sites of the specified amino acid residues.

wherein among the two polypeptides constituting the enzyme having the nitrile hydratase activity before  
15 modification, one is the polypeptide of [E] according to [10] and the other is the polypeptide of [F] according to [10];

[12] the method for modification according to [8], wherein the polypeptide of [A] is the polypeptide of the  
20 following [G];

[G] a polypeptide containing the region as represented by the amino acid sequence  $X_1CXLC_1SC_2X_2X_3X_4X_5$  (wherein C corresponds to cysteine, X to serine or threonine, L to leucine, C<sub>1</sub> to cysteine sulfinic acid (cysteine sulfinic  
25 acid-3-sulfinoalanine), S to serine, and C<sub>2</sub> to cysteine

sulfenic acid (cysteine sulfenic acid-S-hydroxy-cysteine);  
and  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$  and  $X_5$  represent arbitrary amino acid,  
respectively);

[13] the method for modification according to [12],  
5 wherein  $X_1$  is valine,  $X_4$  is tryptophan, and  $X_5$  is proline;

[14] the method for modification according to [13],  
wherein  $X_2$  is tyrosine and  $X_3$  is proline;

[15] the method for modification according to any one  
of [12] to [14], wherein bonding with a metal atom is  
10 located in the region represented by  $X_1CXLC_1SC_2X_2X_3X_4X_5$ ;

[16] the method for modification according to [15],  
wherein the metal atom is a cobalt atom;

[17] a modified enzyme obtained by the method for  
modification according to any one of [1] to [16];

15 [18] a gene encoding the modified enzyme according to  
[17];

[19] a plasmid containing the gene according to [18];

[20] a transformant obtained by transformation of a  
microorganism with the gene according to [18] or the plasmid  
20 according to [19];

[21] a method for production of a modified enzyme,  
comprising the step of recovering a modified enzyme from a  
culture obtained from cultivating the transformant according  
to [20], the cultivated cells or a product obtained from the  
25 processing of the culture or the cultivated cells; and

[22] a method for production of an amide compound, characterized in comprising the step of bringing the modified enzyme that is obtained from a culture obtained from cultivating the transformant according to [20], the  
5 cultivated cells or a product obtained from the processing of the culture or the cultivated cells, or the method for production according to [21], into contact with a nitrile compound in a solvent to convert the nitrile compound to a corresponding amide compound.

10 The invention provides the amino acid sequence and the base sequence of the gene of a *Pseudonocardia thermophila*-derived nitrile hydratase which has a novel mutation point that does not change the fundamental function of nitrile hydratase. It also provides a plasmid containing the gene,  
15 a transformant containing the plasmid, a method for producing the enzyme using the transformant, and a method for processing a nitrile compound by using the transformant to produce a corresponding amide compound.

Further, the invention provides a method for modifying  
20 an enzyme having the nitrile hydratase activity before modification, by using the means characterized in changing the stereostructure of the enzyme having the nitrile hydratase activity. According to the method for modification using this means, an effect may be achieved  
25 that one or more properties selected from the enzyme

activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against the substrate, stability against the product or the like is modified. Furthermore, the invention provides a nitrile hydratase having a novel mutation point,  
5 and a gene encoding the polypeptide chain constituting the enzyme. Also, the invention provides a plasmid containing the gene, a transformant containing the gene or the plasmid, a method for producing the enzyme using the transformant, and a method for processing a nitrile compound by using the  
10 transformant to produce a corresponding amide compound.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a restriction endonuclease cleavage map of  
15 a plasmid pPT-DB1 extracted from MT10822 (Reference Example 1, Examples 1 and 83).

Fig. 2 shows a restriction endonuclease cleavage map of a plasmid PJ1H-DB1 constructed in order to activate and express the *Rhodococcus rhodochrous* strain J1-derived  
20 nitrile hydratase (Example 84).

The abbreviations used in Fig. 1 and Fig. 2 have the following meanings.

bla indicates the ORF encoding  $\beta$ -lactamase.

25 ColE1-ori indicates the replication-starting site in a

ColE1 system.

lacZ indicates the promoter and operator region in pUC18-derived lactose operon.

NH $\alpha$  indicates the ORF encoding the  $\alpha$ -subunit of  
5 *Pseudonocardia thermophila*-derived nitrile hydratase.

NH $\beta$  indicates the ORF encoding the  $\beta$ -subunit of  
*Pseudonocardia thermophila*-derived nitrile hydratase.

P16 indicates the ORF encoding a protein characterized  
in being involved in the activation of *Pseudonocardia*  
10 *thermophila*-derived nitrile hydratase.

nhhA indicates the ORF encoding the  $\alpha$ -subunit of  
*Rhodococcus rhodochrous* strain J1-derived nitrile hydratase.

nhhB indicates the ORF encoding the  $\beta$ -subunit of  
*Rhodococcus rhodochrous* strain J1-derived nitrile hydratase.

15

#### BEST MODE FOR CARRYING OUT THE INVENTION

Now, the invention will be explained in more detail.

The nitrile hydratase of the invention is obtained by  
20 introducing mutation into an enzyme having the nitrile  
hydratase activity, and for example, it is obtained by  
introducing mutation into *Pseudonocardia thermophila*-derived  
nitrile hydratase. Specifically, such nitrile hydratase  
fundamentally consisted of the amino acid sequences as set  
25 forth in SEQ ID NOs: 1 and 2 in the Sequence Listing, in

which amino acid at one or more predetermined sites in the amino acid sequence is substituted by another amino acid. Thus, the invention comprises a nitrile hydratase having as its constituent the  $\alpha$ -subunit represented by the sequence of 205 amino acids as set forth in SEQ ID NO: 1 in the Sequence Listing, in which at least one amino acid in the amino acid sequence is substituted by another amino acid; a nitrile hydratase having as its constituent the  $\beta$ -subunit represented by the sequence of 438 amino acids in total which constitute the  $\beta$ -subunit represented by the sequence of 233 amino acids as set forth in SEQ ID NO: 2 in the Sequence Listing, in which at least one amino acid in the amino acid sequence is substituted by another amino acid; and a nitrile hydratase having both of the above-mentioned substitution cases as its constituent.

According to the invention, the specific amino acid sequence used for the nitrile hydratase obtained by introducing mutation into *Pseudonocardia thermophila*-derived nitrile hydratase, includes the following:

(a-0) the amino acid sequence of the  $\alpha$ -subunit of SEQ ID NO: 1;

(a-1) an amino acid sequence having mutation in which at least one amino acid of the 36<sup>th</sup>, 71<sup>st</sup>, 148<sup>th</sup> and 204<sup>th</sup> amino acids in the amino acid sequence of the  $\alpha$ -subunit as set forth in SEQ ID NO: 1 is substituted by another amino



acid;

(a-2) an amino acid sequence having mutation in which at least one amino acid of the 6<sup>th</sup>, 19<sup>th</sup>, 38<sup>th</sup>, 77<sup>th</sup>, 90<sup>th</sup>, 102<sup>nd</sup>, 106<sup>th</sup>, 126<sup>th</sup>, 130<sup>th</sup>, 142<sup>nd</sup>, 146<sup>th</sup>, 187<sup>th</sup>, 194<sup>th</sup> and 203<sup>rd</sup> amino acids in the amino acid sequence of the  $\alpha$ -subunit as set forth in SEQ ID NO: 1 is substituted by another amino acid;

(b-0) the amino acid sequence of the  $\beta$ -subunit as set forth in SEQ ID NO: 2 in the Sequence Listing;

(b-1) an amino acid sequence having mutation in which at least one amino acid of the 10<sup>th</sup>, 32<sup>nd</sup>, 37<sup>th</sup>, 41<sup>st</sup>, 46<sup>th</sup>, 48<sup>th</sup>, 51<sup>st</sup>, 72<sup>nd</sup>, 118<sup>th</sup>, 127<sup>th</sup>, 146<sup>th</sup>, 160<sup>th</sup>, 186<sup>th</sup> and 217<sup>th</sup> amino acids in the amino acid sequence of the  $\beta$ -subunit as set forth in SEQ ID NO: 2 is substituted by another amino acid; and

(b-2) an amino acid sequence having mutation in which at least one amino acid of the 20<sup>th</sup>, 21<sup>st</sup>, 108<sup>th</sup>, 200<sup>th</sup>, and 212<sup>th</sup> amino acids in the amino acid sequence of the  $\beta$ -subunit as set forth in SEQ ID NO: 2 is substituted by another amino acid.

Each of the above-mentioned mutations at least makes it possible to maintain the nitrile hydratase activity before modification.

According to the invention, the nitrile hydratase obtained by introducing mutation into *Pseudonocardia*

*thermophila*-derived nitrile hydratase, consists of the following constituents having an amino acid sequence selected from the above (a-0) to (b-2):

(A-1) nitrile hydratase in which the  $\alpha$ -subunit has the  
5 amino acid sequence containing the mutation of (a-1) above;

(A-2) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutations of (a-1) and (a-2) above;

(B-1) nitrile hydratase in which the  $\beta$ -subunit has the  
10 amino acid sequence containing the mutation of (b-1) above;

(B-2) nitrile hydratase in which the  $\beta$ -subunit has the amino acid sequence containing the mutations of (b-1) and (b-2) above;

(A-3) nitrile hydratase in which the  $\alpha$ -subunit has the  
15 amino acid sequence containing the mutation of (a-1) above and the  $\beta$ -subunit has the amino acid sequence of (b-0) above;

(A-4) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutation of (a-1) above  
20 and the  $\beta$ -subunit has the amino acid sequence containing the mutation of (b-1) above;

(A-5) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutation of (a-1) above and the  $\beta$ -subunit has the amino acid sequence containing the  
25 mutation of (b-2) above;

(A-6) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutation of (a-1) above and the  $\beta$ -subunit has the amino acid sequence containing the mutations of (b-1) and (b-2) above;

5 (A-7) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutations of (a-1) and (a-2) above and the  $\beta$ -subunit has the amino acid sequence of (b-0) above;

10 (A-8) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutations of (a-1) and (a-2) above and the  $\beta$ -subunit has the amino acid sequence containing the mutation of (b-1) above;

15 (A-9) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutations of (a-1) and (a-2) above and the  $\beta$ -subunit has the amino acid sequence containing the mutation of (b-2) above;

20 (A-10) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutations of (a-1) and (a-2) above and the  $\beta$ -subunit has the amino acid sequence containing the mutations of (b-1) and (b-2) above;

(B-3) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence of (a-0) above and the  $\beta$ -subunit has the amino acid sequence of (b-1);

25 (B-4) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence of (a-0) above and the  $\beta$ -subunit has the

amino acid sequence containing the mutations of (b-1) and (b-2) above; and

(B-5) nitrile hydratase in which the  $\alpha$ -subunit has the amino acid sequence containing the mutation of (a-2) above  
5 and the  $\beta$ -subunit has the amino acid sequence containing the mutations of (b-1) and (b-2) above.

In addition, with respect to the amino acids other than the above-mentioned specific mutation sites, substitution, insertion and deletion of amino acids may occur within the  
10 scope of not impairing the nitrile hydratase activity intended by mutation at the specific sites.

According to the invention, the nitrile hydratase gene obtained by introducing mutation into *Pseudonocardia thermophila*-derived nitrile hydratase contains the gene  
15 encoding the  $\alpha$ -subunit of nitrile hydratase and the gene encoding the  $\beta$ -subunit of nitrile hydratase.

As a family of genes involved in the invention, mention may be made of those subjected to introduction of mutation into the gene of *Pseudonocardia thermophila*-derived nitrile  
20 hydratase, and they include the gene encoding the amino acid sequence of the  $\alpha$ -subunit; the gene encoding the  $\beta$ -subunit; and the nitrile hydratase gene having both of the gene encoding the  $\alpha$ -subunit and the gene encoding the  $\beta$ -subunit.

More specifically, the following can be mentioned.

25 (G-1) a gene having the base sequence that codes for

the amino acid sequence having the mutation of the above-mentioned (a-1);

(G-2) a gene having the base sequence that codes for the amino acid sequence having the mutations of the above-mentioned (a-1) and (a-2);

(G-3) a gene having the base sequence that codes for the amino acid sequence having the mutation of the above-mentioned (b-1);

(G-4) a gene having the base sequence that codes for the amino acid sequence having the mutations of the above-mentioned (b-1) and (b-2); and

(G-3) a gene having the base sequence that codes for any one of the nitrile hydratases of the above-mentioned (A-1) to (B-4).

As the base sequence encoding the amino acid sequence of the  $\alpha$ -subunit of SEQ ID NO: 1 which serves as the bases for the above-mentioned mutations, the base sequence of SEQ ID NO: 3 is preferable. Also, as the base sequence encoding the amino acid sequence of the  $\beta$ -subunit of SEQ ID NO: 2 which serves as the bases for the above-mentioned mutations, the base sequence of SEQ ID NO: 4 is preferable.

For example, the mutation of (a-1) above which is based on SEQ ID NO: 3 can be obtained by substituting at least one base sequence among the 106<sup>th</sup> to 108<sup>th</sup>, 211<sup>th</sup> to 213<sup>th</sup>, 442<sup>nd</sup> to 444<sup>th</sup>, and 610<sup>th</sup> to 612<sup>th</sup> of the base sequence of SEQ ID NO: 3,

by another base sequence.

Further, the mutation of (a-2) above which is based on SEQ ID NO: 3 can be obtained by the substitution of a portion of the base sequence obtained by substituting at least one base sequence among the 16<sup>th</sup> to 18<sup>th</sup>, 55<sup>th</sup> to 57<sup>th</sup>, 112<sup>th</sup> to 114<sup>th</sup>, 229<sup>th</sup> to 231<sup>st</sup>, 268<sup>th</sup> to 270<sup>th</sup>, 304<sup>th</sup> to 306<sup>th</sup>, 316<sup>th</sup> to 318<sup>th</sup>, 376<sup>th</sup> to 378<sup>th</sup>, 388<sup>th</sup> to 390<sup>th</sup>, 424<sup>th</sup> to 426<sup>th</sup>, 436<sup>th</sup> to 438<sup>th</sup>, 559<sup>th</sup> to 561<sup>st</sup>, 580<sup>th</sup> to 582<sup>nd</sup>, and 607<sup>th</sup> to 609<sup>th</sup> of the base sequence of SEQ ID NO: 3, by another base sequence.

Meanwhile, the mutation of (b-1) above which is based on SEQ ID NO: 4 can be obtained by the substitution of a portion of the base sequence obtained by substituting at least one base sequence among the 28<sup>th</sup> to 30<sup>th</sup>, 94<sup>th</sup> to 96<sup>th</sup>, 109<sup>th</sup> to 111<sup>th</sup>, 121<sup>st</sup> to 123<sup>rd</sup>, 136<sup>th</sup> to 138<sup>th</sup>, 142<sup>nd</sup> to 144<sup>th</sup>, 151<sup>st</sup> to 153<sup>rd</sup>, 214<sup>th</sup> to 216<sup>th</sup>, 352<sup>nd</sup> to 354<sup>th</sup>, 379<sup>th</sup> to 381<sup>st</sup>, 436<sup>th</sup> to 438<sup>th</sup>, 478<sup>th</sup> to 480<sup>th</sup>, 556<sup>th</sup> to 558<sup>th</sup>, and 649<sup>th</sup> to 651<sup>st</sup> of the base sequence of SEQ ID NO: 4, by another base sequence.

Further, the mutation of (b-2) above which is based on SEQ ID NO: 4 can be obtained by the substitution of a portion of the base sequence obtained by substituting at least one base sequence among the 58<sup>th</sup> to 60<sup>th</sup>, 61<sup>st</sup> to 63<sup>rd</sup>, 322<sup>nd</sup> to 324<sup>th</sup>, 598<sup>th</sup> to 600<sup>th</sup>, and 634<sup>th</sup> to 636<sup>th</sup> of the base sequence of SEQ ID NO: 4, by another base sequence.

These substitutions are carried out within the scope such that the activity of the nitrile hydratase into which at least one of the  $\alpha$ - and  $\beta$ -subunits encoded by the respective genes is combined and inserted, maintains or  
5 improves the state before substitution. Also, there is no particular limitation on the means for introducing mutation.

With regard to the sites other than the mutation sites of (a-1), (a-2), (b-1) and (b-2) above in the nitrile hydratase genes of the invention, they may have additional  
10 mutations involving substitution, insertion or deletion of bases, within the scope that the gene can function as the template for the protein having the nitrile hydratase activity.

Such additional mutations may be exemplified by the  
15 following. Even in the case where transcription and translation are carried out using a gene having a certain base sequence as the template, depending on conditions such as the type of the host cell incorporating the gene, the components or composition of the nutrition medium used in  
20 cultivation, the temperature or pH during cultivation, or the like, modification by enzymes within the host cell after expression of the gene can lead to production of mutants in which one or two or more amino acids near the N-terminal in the Sequence Listing are deleted, or one or two or more  
25 amino acids are newly added at the N-terminal, with the

intended enzymatic action being still maintained. For this reason, such mutant nitrile hydratases are also to be included in the scope of invention.

Meanwhile, the plasmid for the production of the  
5 nitrile hydratase of the invention can be prepared by using the nitrile hydratase genes. Specific examples thereof may include the following:

(P-1) a plasmid having the base sequence that codes for the amino acid sequence having the mutation of the above-  
10 mentioned (a-1);

(P-2) a plasmid having the base sequence that codes for the amino acid sequence having the mutations of the above-mentioned (a-1) and (a-2);

(P-3) a plasmid having the base sequence that codes for  
15 the amino acid sequence having the mutation of the above-mentioned (b-1);

(P-4) a plasmid having the base sequence that codes for the amino acid sequence having the mutations of the above-mentioned (b-1) and (b-2); and

20 (P-5) a plasmid having the base sequences that code for several nitrile hydratases of the above-mentioned (A-1) to (B-4).

Furthermore, the transformant or strain of the invention is obtained by transforming an arbitrary host cell.  
25 using the plasmid. The method for production of nitrile



hydratase of the invention comprises a step of producing nitrile hydratase by cultivating the transformant or strain. Also, the method for production of amide compound of the invention comprises a step of bringing a culture obtained by the cultivation of such transformant or strain producing nitrile hydratase, the cultivated cells or a product obtained by processing the cultivated cells into contact with a nitrile compound in a medium to produce a corresponding amide compound.

Next, the method for modifying the enzyme having the nitrile hydratase activity of the invention will be explained in detail.

According to the invention, the nitrile hydratase activity means the activity of hydrating a nitrile compound into a corresponding amide compound. The enzyme having this activity is in general characterized in that it comprises two types of polypeptide chains called the  $\alpha$ -subunit and the  $\beta$ -subunit as the constituent, and the nitrile hydratase gene refers to the two amino acid sequences having the feature of forming those two polypeptide chains or to the base sequences constituting the two ORFs having the feature of encoding the amino acid sequences.

To illustrate specifically on *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase (see SEQ ID NOS: 98 and 99 in the Sequence Listing, Patent Document 3,

Non-patent Document 4, and PDB ID NO: 1IRE) as an example, the polypeptide consisting of the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing as well as Chain A in the stereostructure of nitrile hydratase as set forth in PDB ID NO: 1IRE is the  $\alpha$ -subunit; and the polypeptide consisting of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing as well as Chain B in the stereostructure of nitrile hydratase as set forth in PDB ID NO: 1IRE is the  $\beta$ -subunit. Further, the ORF composed of the base sequence as set forth in SEQ ID NO: 100 in the Sequence Listing and the ORF composed of the base sequence as set forth in SEQ ID NO: 101 in the Sequence Listing are referred to as the nitrile hydratase gene.

In addition, in the case of *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase (see SEQ ID NO: 104 in the Sequence Listing, Patent Document 2 and Non-patent Document 1), the polypeptide consisting of the amino acid sequence that is encoded by the ORF formed by the 704<sup>th</sup> to 1315<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing is the  $\alpha$ -subunit; the polypeptide consisting of the amino acid sequence that is encoded by the ORF formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing is the  $\beta$ -subunit; and the two ORFs encoded by the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing are the

nitrile hydratase gene.

The method for modification as used in the invention means a method directed to changing the stereostructure of an enzyme having the nitrile hydratase activity before  
5 modification for the purpose of changing one or more properties among the enzymatic activity, substrate specificity,  $V_{\max}$ ,  $K_m$ , thermal stability, stability against the substrate and stability against the product, without impairing the original nitrile hydratase activity. As the  
10 feature of the method, it can be mentioned that the method comprises a step of specifying, based on an interpretation of the stereostructure, the region forming a cavity through which a substrate passes from the outside of the enzyme toward the active center or a product passes from the active  
15 center toward the outside of the enzyme, and/or the region forming an associative interface between the  $\alpha$ -subunit and the  $\beta$ -subunit which is involved in the formation of dimers or an interface which is involved in the association of dimers, and applying alterations such as substitution,  
20 insertion or deletion at one or more amino acids in the amino acid sequence which correspond to the amino acid residues that are present in the regions.

To illustrate specifically on *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase (see SEQ ID  
25 NOs: 98 and 99 in the Sequence Listing, Patent Document 3,

Non-patent Document 4, and PDB ID NO: 1IRE) as an example, for the amino acid residues corresponding to those present in the region forming a cavity through which a substrate passes from the outside of the enzyme toward the active center or a product passes from the active center toward the outside of the enzyme, mention may be made of the amino acid residues forming the region that corresponds to the 1<sup>st</sup> helix and 2<sup>nd</sup> helix as counted from the N-terminal of Chain B and the loop portions inserted in the helices in the nitrile hydratase stereostructure as set forth in PDB ID NO: 1IRE; the amino acid residues whose side-chain front-end heavy atoms are located within 5Å of radius in the respective stereostructures having as the point center, each of the side-chain front-end heavy atoms of the four amino acid residues which correspond to the 89<sup>th</sup> amino acid residue glutamine and the 165<sup>th</sup> amino acid residue glutamic acid as counted from the N-terminal of Chain A, and to the 37<sup>th</sup> amino acid residue phenylalanine and the 48<sup>th</sup> amino acid residue leucine as counted from the N-terminal of Chain B in the nitrile hydratase stereostructure as set forth in PDB ID NO: 1IRE; the amino acid residues corresponding to the region extending from the 36<sup>th</sup> threonine to 48<sup>th</sup> asparagine of the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing, to the region extending from the 31<sup>st</sup> lysine to the 51<sup>st</sup> phenylalanine and to the region extending

from the 112<sup>th</sup> lysine to 127<sup>th</sup> leucine of the amino acid  
sequence as set forth in SEQ ID NO: 99 in the Sequence  
Listing; and the amino acid residues corresponding to the  
37<sup>th</sup>, 40<sup>th</sup>, 41<sup>st</sup>, 46<sup>th</sup>, 48<sup>th</sup>, 51<sup>st</sup>, 61<sup>st</sup>, 72<sup>nd</sup>, 112<sup>th</sup>, 118<sup>th</sup> and  
5 127<sup>th</sup> of the amino acid sequence as set forth in SEQ ID NO:  
99 in the Sequence Listing; or the like.

Furthermore, for the amino acid residues corresponding  
to those present in the region forming an associative  
interface between the  $\alpha$ -subunit and the  $\beta$ -subunit which is  
10 involved in the formation of dimers or an interface which is  
involved in the association of dimers, mention may be made  
of the amino acid residues corresponding to the 2<sup>nd</sup> helix as  
counted from the N-terminal of Chain A and the 2<sup>nd</sup> helix as  
counted from the N-terminal of Chain B in the nitrile  
15 hydratase stereostructure as set forth in PDB ID NO: 1IRE,  
to the region extending from the 36<sup>th</sup> threonine to the 48<sup>th</sup>  
asparagine of the amino acid sequence as set forth in SEQ ID  
NO: 98 in the Sequence Listing, and to the region extending  
from the 112<sup>th</sup> lysine to the 127<sup>th</sup> leucine of the amino acid  
20 sequence as set forth in SEQ ID NO: 99 in the Sequence  
Listing; the amino acid residues corresponding to the 36<sup>th</sup>,  
71<sup>st</sup>, 148<sup>th</sup>, 188<sup>th</sup> and 204<sup>th</sup> of the amino acid sequence as set  
forth in SEQ ID NO: 98 in the Sequence Listing; the amino  
acid residues corresponding to the 10<sup>th</sup>, 32<sup>nd</sup>, 33<sup>rd</sup>, 112<sup>th</sup>,  
25 118<sup>th</sup>, 127<sup>th</sup>, 146<sup>th</sup>, 150<sup>th</sup>, 160<sup>th</sup>, 168<sup>th</sup>, 171<sup>st</sup>, 176<sup>th</sup>, 186<sup>th</sup>,

217<sup>th</sup> and 218<sup>th</sup> of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing; or the like.

In addition, as the method for specifying, among the amino acid residues constituting the region that forms a cavity through which a substrate passes from the outside of the enzyme toward the active center or a product passes from the active center toward the outside of the enzyme, the amino acid residues whose alteration leads to a change in the cavity size and further controls the easiness or difficulty in passing of the substrate/product, mention may be made of a method for specifying the four amino acid residues which correspond to the 89<sup>th</sup> amino acid glutamine (A89Q) and the 165<sup>th</sup> amino acid glutamic acid (A165E) as counted from the N-terminal of Chain A, and to the 37<sup>th</sup> amino acid phenylalanine (B37F) and the 48<sup>th</sup> amino acid leucine (B48L) as counted from the N-terminal of Chain B in the nitrile hydratase stereostructure as set forth in PDB ID NO: 1IRE; a method for specifying the amino acid residues which effect a change in at least one of d1 to d5, when the shortest distance between the heavy atoms of the amino acid residue corresponding to A165E and of the amino acid residue corresponding to B48L is designated as d1; the shortest distance between the heavy atoms of the amino acid residue corresponding to A89Q and of the amino acid residue corresponding to B48L as d2; the shortest distance between

the heavy atoms of the amino acid residue corresponding to B37F and of the amino acid residue corresponding to B48L is designated as d3; the shortest distance between the heavy atoms of the amino acid residue corresponding to A165E and  
5 of the amino acid residue corresponding to B37F as d4; and the shortest distance between the heavy atoms of the amino acid residue corresponding to A89Q and of the amino acid residue corresponding to B37F as d5; a method for specifying the amino acid residues which effect a change in at least  
10 one of the above-described d1 to d5, or for specifying the amino acid residues which effect a change in at least one of the above-described d1 to d3; or the like.

Therefore, the invention includes any method for modification, comprising a step of applying alterations such  
15 as substitution, insertion or deletion at amino acids in the amino acid sequence which correspond to at least one amino acid residues as specified in the above-described manner in a subject nitrile hydratase (*Pseudonocardia thermophila* JCM3095-derived one may be mentioned as a representative  
20 example).

Furthermore, the invention also includes the method for modification by aligning a nitrile hydratase (for example, a *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase) derived from a biological species other than *Pseudonocardia*  
25 *thermophila* JCM3095 with the stereostructure or the amino

acid sequence of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase, finding the amino acid residues corresponding to the above-mentioned amino acid residues and altering the amino acids in the corresponding amino acid sequences.

In carrying out the subject matters described above, the means used to implementing alignment based on the stereostructure or amino acid sequence is not particularly limited. However, as exemplary means for the alignment of amino acid sequence, software for genomic sequence interpretation such as DNASIS (manufactured by Hitachi Software Engineering Co., Ltd.) or a free software ClustalW or BLAST may be mentioned; and as exemplary means for the modeling of stereostructure based on the amino acid sequence alignment, software for predicting protein stereostructure such as Modeler and Homology (products by Accelrys Software, Inc.) may be mentioned.

Speaking of an example, in the case of a *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase (see SEQ ID NO: 104 in the Sequence Listing, Patent Document 2 and Non-patent Document 1), the results of alignment show that the amino acid residue corresponding to the amino acid residue: Leu which corresponds to the 48<sup>th</sup> of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing is the amino acid residue: Trp corresponding to the 48<sup>th</sup> of the



amino acid sequence which is encoded by the ORF formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing; and the amino acid residue corresponding to the amino acid residue: Phe which

5 corresponds to the 51<sup>st</sup> of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing is the amino acid residue: Ser corresponding to the 51<sup>st</sup> of the amino acid sequence which is encoded by the ORF formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in  
10 the Sequence Listing. The results are consistent with the alignment results based on amino acid sequence and the alignment results based on stereostructure. The invention also includes the method for modification by altering any one or both of the amino acids in the amino acid sequence  
15 which corresponds to these two amino acid residues.

Moreover, in carrying out the above-described method for modification, the means for introducing mutation to alter amino acids in the amino acid sequence corresponding to amino acid residues is not particularly limited. But, an  
20 example may include a method for introducing mutation of substituting an amino acid in the amino acid sequence with another amino acid by means of recombinant gene technology.

Furthermore, with respect to any changes in the amino acid sequence or base sequence resulting from a mutation  
25 additionally introduced in addition to the intentionally

introduced mutation, substitution, insertion or deletion of an amino acid or a base may occur within the scope of not impairing the desired nitrile hydratase activity achieved by the intended introduction of mutation.

5       Such additionally introduced mutation may be exemplified by the following. Even in the case where transcription and translation are carried out using a gene having a certain base sequence as the template, depending on conditions such as the type of the host cell incorporating  
10   the gene, the components or composition of the nutrition medium used in cultivation, the temperature or pH during cultivation, or the like, modification by enzymes inside the host cell after expression of the gene can lead to production of mutants in which one or two or more amino  
15   acids near the N-terminal in the Sequence Listing are deleted, or one or two or more amino acids are newly added at the N-terminal, with the initial enzymatic action being still maintained. For this reason, such method for modification resulting in mutant nitrile hydratases is also  
20   to be included in the scope of invention.

As exemplary nitrile hydratases before modification which serve as the subject for the method for modification according to the invention, a *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase and a *Pseudonocardia*  
25   *thermophila* JCM3095-derived nitrile hydratase may be

mentioned. More specifically, mention may be made of a nitrile hydratase comprising as its constituents the polypeptide chain formed by an amino acid sequence which is homologous to the amino acid sequence encoded by the ORF which is formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing and the polypeptide chain formed by an amino acid sequence which is homologous to the amino acid sequence encoded by the ORF which is formed by the 704<sup>th</sup> to 1315<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing; a nitrile hydratase comprising as its constituents the polypeptide chain formed from the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing and the polypeptide chain formed from the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing.

The nitrile hydratase before modification which serve as the subject for the method for modification according to the invention may also include a nitrile hydratase comprising as its constituents the polypeptide formed from an amino acid sequence which shows homology of 40% or greater with the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing and the polypeptide formed from an amino acid sequence which shows homology of 25% or greater with the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing.

Examples of the polypeptide formed from an amino acid sequence which shows homology of 40% or greater with the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing may include the polypeptide formed from the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing; the polypeptide formed from an amino acid sequence in which arbitrary alteration such as substitution, insertion or deletion is applied to at least one site of the amino acid sequence as set forth in SEQ ID NO: 98; the polypeptide formed from an amino acid sequence in which at least one amino acid selected from the 6<sup>th</sup>, 19<sup>th</sup>, 38<sup>th</sup>, 77<sup>th</sup>, 90<sup>th</sup>, 102<sup>nd</sup>, 106<sup>th</sup>, 126<sup>th</sup>, 130<sup>th</sup>, 142<sup>nd</sup>, 146<sup>th</sup>, 187<sup>th</sup>, 194<sup>th</sup> and 203<sup>rd</sup> amino acids in the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing is substituted by another amino acid; the polypeptide formed from an amino acid sequence that is homologous to the amino acid sequence encoded by the ORF which is formed by the 704<sup>th</sup> to 1315<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing; or the like.

Further, the polypeptide formed from an amino acid sequence which shows homology of 40% or greater with the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing is characterized, in some cases, in that it is a polypeptide containing a region represented by

$X_1CXLC_1SC_2X_2X_3X_4X_5$  (wherein C corresponds to cysteine, X to

serine or threonine, L to leucine, C<sub>1</sub> to cysteine sulfinic acid (cysteine sulfinic acid·3-sulfinoalanine), S to serine, and C<sub>2</sub> to cysteine sulfenic acid (cysteine sulfenic acid·S-hydroxy-cysteine); and X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, X<sub>4</sub> and X<sub>5</sub> represent

5 arbitrary amino acid, respectively) in the sequence.

Additionally, the polypeptide is also characterized, in some cases, in that X<sub>1</sub> is valine, X<sub>4</sub> is tryptophan, and X<sub>5</sub> is proline. Again, the polypeptide is also characterized, in some cases, in that X<sub>2</sub> is tyrosine and X<sub>3</sub> is proline.

10 In the above-mentioned cases, the polypeptide is in some cases characterized in being bonded to a metal atom via the region represented by X<sub>1</sub>CXLC<sub>1</sub>SC<sub>2</sub>X<sub>2</sub>X<sub>3</sub>X<sub>4</sub>X<sub>5</sub>. In addition, this metal is in some cases characterized in being cobalt.

Examples of the polypeptide formed from an amino acid  
15 sequence which shows homology of 25% or greater with the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing may include the polypeptide formed from the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing; the polypeptide formed from an amino acid  
20 sequence in which arbitrary alteration such as substitution, insertion or deletion is applied to at least one site of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing; the polypeptide formed from an amino acid sequence in which at least one amino acid selected from the  
25 20<sup>th</sup>, 21<sup>st</sup>, 108<sup>th</sup>, 200<sup>th</sup> and 212<sup>th</sup> amino acids in the amino

acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing is substituted by another amino acid; the polypeptide formed from an amino acid sequence that is homologous to the amino acid sequence encoded by the ORF which is formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing; or the like.

Speaking of an example, in the case of *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase, since it is a nitrile hydratase comprising as its constituents the polypeptide formed from the amino acid sequence that is encoded by the ORF which is formed by the 704<sup>th</sup> to 1315<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing, and the polypeptide formed from the amino acid sequence that is encoded by the ORF which is formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence Listing, it is included in the scope of nitrile hydratase before modification, which is the subject of the method for modification according to the invention.

Also, since *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase is a nitrile hydratase comprising as its constituents the polypeptide formed from the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing and the polypeptide formed from the amino acid

sequence as set forth in SEQ ID NO: 99 in the Sequence Listing, it is also included in the scope of nitrile hydratase before modification, which is the subject of the method for modification according to the invention.

5           Furthermore, as an example of the nitrile hydratase derived from *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase, mention may be made of a nitrile hydratase satisfying any one or both in the cases of a nitrile hydratase in which, among the constituents of  
10 *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase, the polypeptide formed from the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing is replaced by the polypeptide formed from an amino acid sequence in which arbitrary alteration such as substitution, insertion  
15 or deletion is applied to at least one site of the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing, or by the polypeptide formed from an amino acid sequence in which at least one amino acid of the 6<sup>th</sup>, 19<sup>th</sup>, 38<sup>th</sup>, 77<sup>th</sup>, 90<sup>th</sup>, 102<sup>nd</sup>, 106<sup>th</sup>, 126<sup>th</sup>, 130<sup>th</sup>, 142<sup>nd</sup>, 146<sup>th</sup>, 187<sup>th</sup>,  
20 194<sup>th</sup> and 203<sup>rd</sup> amino acids in the amino acid sequence as set forth in SEQ ID NO: 98 in the Sequence Listing is substituted by another amino acid; and a nitrile hydratase in which the polypeptide formed from the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing is  
25 replaced by the polypeptide formed from an amino acid

sequence in which arbitrary alteration such as substitution, insertion or deletion is applied to at least one site of the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing, or by the polypeptide formed from an amino acid sequence in which at least one amino acid of the 20<sup>th</sup>, 21<sup>st</sup>, 108<sup>th</sup>, 200<sup>th</sup> and 212<sup>th</sup> amino acids in the amino acid sequence as set forth in SEQ ID NO: 99 in the Sequence Listing is substituted by another amino acid.

A nitrile hydratase derived from *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase is also included in the scope of nitrile hydratase before modification, which is the subject of the method for modification according to the invention.

According to the invention, a modified enzyme means a nitrile hydratase obtained by carrying out the method for modification using an enzyme having the nitrile hydratase activity as the subject of the method. An example thereof may include a modified enzyme characterized in being obtained by changing the character of a nitrile hydratase before modification by using the above-described methods for modification.

For the change of characters, as compared with a nitrile hydratase before modification, mention may be made of change in one or more properties such as the substrate specificity,  $V_{max}$ ,  $K_m$ , thermal stability, stability against



the substrate (for example, an arbitrary nitrile compound which can be converted to a corresponding amide compound upon the action of the enzyme as a catalyst), stability against the product (for example, the corresponding amide compound obtained by converting an arbitrary nitrile compound upon the action of the enzyme as a catalyst) or the like. Specific examples may include the following:

(1) It becomes easy to take a nitrile compound with relatively larger bulkiness as the substrate.

(2) It becomes easy to take a nitrile compound with relatively smaller bulkiness as the substrate

(3)  $V_{\max}$  in the case of taking an arbitrary nitrile compound as the substrate, increases

(4)  $K_m$  in the case of taking an arbitrary nitrile compound as the substrate, decreases.

(5) The rate of irreversible deactivation in the case of exposing the enzyme to an arbitrary amount of heat, decreases.

(6) The rate of irreversible deactivation in the case of exposing the enzyme to the substrate at an arbitrary concentration, decreases.

(7) The rate of reaction inhibition due to the presence of the product at an arbitrary concentration during the reaction, decreases.

(8) The rate of irreversible deactivation in the case

of exposing the enzyme to the product at an arbitrary concentration, decreases, or the like.

As an index for the change of character resulting from carrying out the method for modification on a nitrile  
5 hydratase before modification according to the invention, the change in the substrate specificity may be mentioned as one representative example. When a modified enzyme as obtained according to the method for modifying the invention is one that has undergone a change in the substrate  
10 specificity, the modified enzyme is included in the scope of modified enzyme according to the invention.

As the method for observing the change in substrate specificity of thus obtained modified enzyme, mention may be made of a method of carrying out the reaction using a  
15 plurality of nitrile compounds of different bulkiness as the substrate and determining the difference in the quantities of the produced corresponding amide compounds. As an example thereof, the reaction is carried out, on the one hand, using acrylonitrile as the substrate to produce  
20 acrylamide, and on the other hand, using methacrylonitrile as the substrate to produce methacrylamide, and the molar ratio of the two products may be compared. In this case, a modified enzyme which results in a change such that the value of [moles of produced methacrylamide] ÷ [moles of  
25 produced acrylamide] has increased as compared with the

value obtained by an enzyme before modification, can be said to have undergone a change in character to facilitate the reaction using a nitrile compound with larger bulkiness as the substrate. Similarly, a modified enzyme which results in a change such that the value has decreased, can be said to have undergone a change in character to facilitate the reaction using a nitrile compound with smaller bulkiness as the substrate.

In an exemplary study with *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase and a nitrile hydratase derived from *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase, when the process for modification is carried out comprising the step of specifying, according to the interpretation of stereostructure, the region forming a cavity through which a substrate passes from the outside of the enzyme toward the active center or a product passes from the active center toward the outside of the enzyme, and/or the region forming an associative interface between the  $\alpha$ -subunit and the  $\beta$ -subunit which is involved in the formation of dimers or an interface which is involved in the association of dimers, and applying alterations such as substitution, insertion or deletion at one or more amino acids in the amino acid sequence which correspond to the amino acid residues present in these regions, a modified enzyme which has undergone a change in the molar ratio

between acrylamide produced from the reaction using acrylonitrile as the substrate and methacrylamide produced from the reaction using methacrylonitrile as the substrate, as compared with an enzyme before modification, could be  
5 obtained. Since this can be viewed as a change in substrate specificity, that is, a change of characters, the modified enzyme as obtained above is included in the scope of modified enzyme according to the invention.

In a study with *Rhodococcus rhodochrous* strain J1-  
10 derived nitrile hydratase, when the process for modification was carried out by altering the amino acid residue: Trp which corresponds to the 48<sup>th</sup> of the amino acid sequence encoded by the ORF that is formed by the 1<sup>st</sup> to 690<sup>th</sup> of the base sequence as set forth in SEQ ID NO: 104 in the Sequence  
15 Listing to another amino acid residue, the enzyme showed a change in character to facilitate the reaction using a nitrile compound with larger bulkiness as the substrate, as compared with the subject before modification. Thus, the protein showing the change of character as achieved as such  
20 is included in the scope of modified enzyme according to the invention.

Furthermore, it can be easily deduced that combining the mutation sites of a modified enzyme thus obtained will lead to an additional change of character. Accordingly, a  
25 modified enzyme obtained by such combining of mutation sites

is also included in the scope of modified enzyme according to the invention.

According to the invention, the gene encoding a modified enzyme means the two amino acid sequences having the feature of forming the two types of polypeptide chains which constitute the modified enzyme, or the base sequences forming the two ORFs having the feature of encoding the amino acid sequences.

According to the invention, the plasmid having the feature of containing the gene means the plasmid having the feature of containing in its sequence, the base sequences forming the two ORFs which have the feature of encoding the two amino acid sequences that are characterized in forming the two polypeptide chains constituting the modified enzyme.

The plasmid can have, in addition to the gene according to the invention, a constitution which enables the production of a modified enzyme by means of a transformant or a strain obtained by transforming an arbitrary host cell, such as the regulatory region necessary for the expression of each gene, the region necessary for autonomous replication or the like. The arbitrary host cell as used herein may be exemplified by *Escherichia coli* as used in an embodiment of the below-described Examples, though not intended to be limited to this. Use can be also made of other microorganisms such as *Bacillus* species such as

*Bacillus subtilis*, yeast or *Streptomyces* species.

The regulatory region necessary for expression may include a promoter sequence (including the transcription-regulating operator sequence), a ribosome binding sequence  
5 (SD sequence), a transcription-terminating sequence and the like.

Specific examples of the promoter sequence may include the trp promoter of *E. coli*-derived tryptophan operon, the lac promoter of lactose operon, the lambda-phage-derived PL  
10 promoter and PR promoter, or the *B. subtilis*-derived gluconic acid synthetase promoter (gnt), the alkali protease promoter (apr), the neutral protease promoter (npr), the  $\alpha$ -amylase promoter (amy) and the like. Further, artificially designed or improved sequences such as the tac promoter or  
15 trc promoter can be also used.

The ribosome binding sequence may be exemplified by the *E. coli*-derived sequence, *B. subtilis*-derived sequence or the original sequences of *Rhodococcus* or *Pseudonocardia*, but it is not particularly limited as long as it works in a  
20 desired host cell such as *E. coli* or *B. subtilis*. For example, a consensus sequence comprising a series of 4 or more consecutive bases that are complementary to the 3'-terminal region of 16S ribosome RNA can be prepared by DNA synthesis and used. The transcription-terminating sequence  
25 is not essentially required, but one that is not  $\rho$  factor-

dependent, for example, lipoprotein terminator, trp operon terminator or the like may be used. The sequence order of these regulatory regions on a plasmid is preferably such that the promoter sequence and the ribosome binding sequence are located further upstream to the 5'-terminal than the gene according to the invention, and the transcription-terminating sequence is preferably located further downstream to the 3'-terminal than the gene according to the invention. Also, the base sequences respectively encoding the ORFs which constitute the gene according to the invention may be expressed as individual independent cistrons by means of such regulatory regions, or may be expressed as a polycistron by means of a common regulatory region.

Examples of the plasmid vector satisfying the above requirements may include pBR322, pUC18, pBluescript, pKK223-3 and pSC101, which have a region capable of autonomous replication in *E. coli*, or pUB110, pTZ4, pC194,  $\rho$  11,  $\phi$  1 and  $\phi$  105 which have a region capable of autonomous replication in *B. subtilis*, and the like. Further, examples of the plasmid vector capable of autonomous replication in two or more species of host cells may include pHV14, TRp7, YEp7 and pBS7.

When a nitrile hydratase having the desired activity to express the gene according to the invention is produced, a

protein involved in the activation of nitrile hydratase may be required in some cases.

A protein involved in the activation of nitrile hydratase is a protein having the property such that the presence or absence of the expression of the protein directly controls the activation of nitrile hydratase, and it can be exemplified by the protein involved in the activation of *Pseudonocardia thermophila*-derived nitrile hydratase (nitrile hydratase-activating protein) as described in Patent Document 4. More specifically, the nitrile hydratase-activating protein may be exemplified by one constituted by the sequence of 144 amino acids as presented in the amino acid sequence of SEQ ID NO: 102. Also, the mutant proteins obtained by substitution, insertion or deletion of amino acids in part of the amino acid sequence of SEQ ID NO: 102 are to be included in the nitrile hydratase-activating protein, as long as they are involved in the activation of nitrile hydratase. For such mutant proteins, mention may be made of those having mutation such as substitution, insertion or deletion of one or more amino acids with respect to the amino acid sequence of SEQ ID NO: 102 and maintaining the property involved in the activation of nitrile hydratase.

The gene encoding the nitrile hydratase-activating protein is not particularly limited as long as it is a gene



that codes for the nitrile hydratase-activating protein. Such gene may be exemplified by the gene having the base sequence which codes for the amino acid sequence of SEQ ID NO: 102 and the genes that code for the mutant proteins.

5 Further, as a preferred example of the gene encoding the nitrile hydratase-activating protein, the gene having the base sequence of SEQ ID NO: 103 may be mentioned. Also, if a gene encoding the nitrile hydratase-activating protein functions as the gene encoding the nitrile hydratase-activating protein, even though it is a sequence having substitution, insertion or deletion of one or two or more bases with respect to the base sequence as set forth in SEQ ID NO: 103, then the gene is to be included in the scope of genes encoding the nitrile hydratase-activating protein.

15 As an example of the case of using the gene encoding the nitrile hydratase-activating protein, mention may be made of one in which the ORF of the gene is included into the plasmid of the invention together with the two ORFs forming the gene according to the invention. In this case, the order of these ORFs on the plasmid is not particularly limited. Also, three ORFs may be regulated by the same regulatory region; two of the ORFs may be regulated by the same regulatory region, the other one of the ORFs being regulated by a regulatory region different from the former; 25 or the three ORFs may be each regulated by different

regulatory regions.

For a method of constructing the plasmid of the invention by inserting the gene according to the invention into such vector plasmid, together with those regions  
5 necessary for expression of the activity of the modified enzyme according to the invention, or a method of transforming a desired host cell using the plasmid and a method of producing nitrile hydratase in the transformant, use can be made of those general methods and host cells  
10 known in the art of molecular biology, biological engineering and genetic engineering as described in, for example, "Molecular Cloning, 3<sup>rd</sup> ed." (J. Sambrook et al., Cold Spring Harbor Laboratory Press, 2001) or the like.

According to the invention, the transformant  
15 characterized in being obtained by transformation includes those obtained by transforming a host cell using the gene or plasmid according to the invention. As an example of cultivating the transformant, a method of inoculating the transformant in a culture medium and then incubating it at a  
20 suitable cultivating temperature (in general, 20°C to 50°C) may be mentioned.

Further, when the host cell is a microorganism, LB medium, M9 medium or the like is generally used as the culture medium for cultivating the transformant, and metal  
25 ions may be added to such medium. The metal ion to be added

may be Fe ion and Co ion. The amount of addition may be, for example, 0.1 µg/mL or greater.

For the method for producing a modified enzyme characterized in comprising a step of recovering a modified  
5 enzyme from a culture obtained by cultivating the transformant, the cultivated cells, or a product of processing the culture or cells according to the invention, mention may be made of one comprising a step of recovering the nitrile hydratase activity from the transformant, a  
10 culture of the transformant, or a product of processing the transformant or the culture.

For the method for producing an amide compound characterized in comprising a step of converting a nitrile compound to a corresponding amide compound according to the  
15 invention, mention may be made of one comprising a step of using as the catalyst the nitrile hydratase activity recovered from the transformant, a culture of the transformant, or a product of processing the transformant or the culture, or according to the above-mentioned method for  
20 production, to convert a nitrile compound to a corresponding amide compound.

As an example of the method of using the modified enzyme according to the invention or a transformant having the enzyme activity to process a nitrile compound to produce  
25 a corresponding amide compound, mention may be made of a

method comprising a step of bringing the desired nitrile compound into contact with a purification product or a crude enzyme product of the enzyme, with a culture for the transformant according to the invention, with a transformant  
5 obtained from the culture, or with a product of processing the transformant, in a solvent. The product of processing as used herein includes an extract or a disruption product of the transformant, a post-separation product such as a crude enzyme product obtained by isolating the nitrile  
10 hydratase activated fraction from such extract or disruption product, an enzyme purification product obtained by further purification or the like; and an immobilization product in which the transformant, or an extract, a disruption product or a post-separation product of the transformant is  
15 immobilized by suitable means. The contact temperature is not particularly limited, but it is preferably in the temperature range of not deactivating the nitrile hydratase, and more preferably from the freezing point or higher to 60°C or lower.

20 As the nitrile compound, there is no particular limitation as long as it is a compound which can act as the substrate for the modified enzyme of the invention, and examples may include nitrile compounds having 2 to 4 carbon atoms, such as acetonitrile, propionitrile, acrylonitrile,  
25 methacrylonitrile, n-butyronitrile, isobutyronitrile,

crotononitrile,  $\alpha$ -hydroxyisobutyronitrile and the like. The concentration of such nitrile compound in the solvent is not particularly limited. The reaction temperature is, though not particularly limited, preferably in the range of not  
5 deactivating the nitrile hydratase, and more preferably from the freezing point or higher to 50°C or lower.

Examples as described below will illustrate the invention in more detail, but they are not intended to limit the invention in any way. Also, the HPLC analysis in each  
10 Example and Comparative Example was carried out using Finepak SIL C18-5 (250×4.6  $\phi$  mm; product by JASCO) as the column and a 10 mM aqueous solution of phosphoric acid containing 4 vol% of acetonitrile as the eluent. In addition, acrylamide, acrylonitrile, acrylic acid,  
15 methacrylamide, methacrylonitrile and methacrylic acid were detected by absorbance at 210 nm.

#### EXAMPLES

[Reference Example 1]

20 Construction (1) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Met for the 6<sup>th</sup> Leu in the  $\alpha$ -subunit, introduction of site-specific mutation was performed using a "LA PCR in vitro mutagenesis Kit"  
25 (manufactured by Takara Shuzo Co., Ltd.). Hereinafter, the

"LA PCR in vitro mutagenesis Kit" is simply referred to as the kit. In following Reference Examples, the kit was handled on the basis of the principle thereof and in accordance with the manufacturer's instructions for the kit.

5           10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the cells of MT-10822 were inoculated and incubated  
10           therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid pPT-DB1 was prepared from the cells by  
15           alkaline SDS extraction.

          Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 7  
20           in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which  
25           one PCR cycle comprised thermal denaturation (98°C) for 15

seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out. This assay revealed the production of the amplified DNA products in the both PCR reactions. From each of these PCR reaction mixtures, the excess primers and dNTP were removed using Microcon 100 (manufactured by Takara Shuzo Co., Ltd.), and then TE was added to each of the mixtures to prepare 50 µl each of TE solutions. An annealing solution of 47.5 µl in total containing 0.5 µl of both of the above TE solutions (for the composition of the system, the manufacturer's instructions for the kit were followed) was prepared, and this solution was subjected to annealing by performing thermal denaturation of the solution at 98°C for 10 minutes,

subsequently cooling the solution to 37°C at a constant cooling rate over a period of 60 minutes, and then maintaining it at 37°C for 15 minutes. To thus annealed solution, 0.5 µl of TaKaRa LA Taq was added, and the  
5 solution was heated at 72°C for 3 minutes, thus completing the formation of heterologous double-stranded DNA. This was then subjected to PCR reaction No. 3. For the PCR reaction No. 3, a reaction system of 50 µl in total comprising 50 pmols of an M13 primer M4 (having the sequence as forth in  
10 SEQ ID No: 8 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30  
15 seconds and chain extension (72°C) for 120 seconds. After completion of the PCR reaction No. 3, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, a product by Sigma Corporation; agarose concentration of 0.8% by weight), and  
20 an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product of about 2 kbp. Subsequently, an agarose fragment comprising only a DNA fragment of about 2 kbp was cut out of the agarose gel. The thus-cut agarose  
25 fragment (about 0.1 g) was finely pulverized, suspended in 1



ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was then subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA  
5 fragment. Thus purified DNA fragment was finally dissolved in 10 µl of TE. The amplified DNA fragment of about 2 kbp thus purified was cleaved by means of restriction endonucleases EcoRI and HindIII, and then subjected to phenol/chloroform extraction and ethanol precipitation to  
10 purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10 µl of TE. Likewise, pPT-DB1 was cleaved by means of EcoRI and HindIII, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, a product by Sigma Corporation; agarose  
15 concentration of 0.7%). An agarose fragment comprising only the DNA fragment of about 2.7 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of the TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was  
20 completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10 µl of TE. Thus obtained DNA fragments of about 2 kbp and of about 2.7 kbp were subjected  
25 to DNA ligation, using a DNA ligation kit (manufactured by

Takara Shuzo Co., Ltd.). Then, a competent cell of *E. coli* HB101 (manufactured by Toyobo Co., Ltd.) was transformed with the reaction product to obtain a transformant No. 1.

The conversion and the selectivity were determined in the following manner in the production of the amide compound using the obtained transformant.

In a 500 ml Erlenmeyer flask with baffles, 100 ml of a liquid LB medium comprising 40 µg/ml of ferric sulfate/heptahydrate and 10 µg/ml of cobalt chloride/dihydrate was prepared and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the transformant No. 1 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 130 rpm. The cells were separated from the resulting culture by centrifugation (5,000G × 15 minutes), and then resuspending the cells in 50 ml of physiological saline, followed by another centrifugation (5,000G × 15 minutes). 0.1 g of the cells was suspended in 20 ml of an aqueous solution (pH 7.0) of 50 mM potassium phosphate. To this, 1 ml of acrylonitrile or methacrylonitrile was added, and this mixture was gently stirred at 10°C for 1 hour to react. After completion of the reaction, an analysis of the reaction solution was carried out with HPLC, and it was found that the reaction

solution contained only an amide compound (acrylamide or methacrylamide) of a molar amount corresponding to the amount of the added nitrile compound (acrylonitrile or methacrylonitrile), and that the nitrile compound

5 (acrylonitrile or methacrylonitrile) and the corresponding organic acid (acrylic acid or methacrylic acid) were absent. That is, the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
10 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 1, in which it is known that the 6<sup>th</sup> Leu in  
15 the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Met.

[Table 1]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 1	6 <sup>th</sup> position in $\alpha$ -	Leu	Met	CTG	ATG

	subunit				
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[Reference Example 2]

Construction (2) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Thr for the 6<sup>th</sup> Leu in the  $\alpha$ -  
subunit, using the plasmid DNA pPT-DB1 as the template, the  
plasmid DNA pPT-DB1 was subjected to introduction of site-  
specific mutation in the same manner as in Reference Example  
1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 11 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of an

MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 2 was then obtained in completely the same manner as in Reference Example 1 comprising the PCR reaction No. 2.

The addition rate and the selectivity was determined in the same manner as in Reference Example 1 were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 2, in which it is known that the 6<sup>th</sup> Leu in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Thr.

[Table 2]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 2	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Thr	CTG	ACG

[Reference Example 3]

5 Construction (3) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ala for the 6<sup>th</sup> Leu in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 12 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 3 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 3, in which it is known that the 6<sup>th</sup> Leu in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

10

[Table 3]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 3	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Ala	CTG	GCG

[Reference Example 4]

Construction (4) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15

For the substitution with Val for the 6<sup>th</sup> Leu in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the



plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
5 Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 13 in the Sequence Listing and 50 pmols of an M13 primer M4 (having  
10 the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15  
15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV  
20 (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of  
25 the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 4 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 4, in which it is known that the 6<sup>th</sup> Leu in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

[Table 4]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 4	6 <sup>th</sup> position	Leu	Val	CTG	GTG

	in $\alpha$ - subunit				
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[Reference Example 5]

Construction (5) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Val for the 19<sup>th</sup> Ala in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 14 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 5 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 5, in which it is known that the 19<sup>th</sup> Ala in  
25 the  $\alpha$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Val.

[Table 5]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 5	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG

[Reference Example 6]

5 Construction (6) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Leu for the 38<sup>th</sup> Met in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 15 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 6 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 6, in which it is known that the 38<sup>th</sup> Met in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

10 [Table 6]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 6	38 <sup>th</sup> position in $\alpha$ -subunit	Met	Leu	ATG	TTG

[Reference Example 7]

Construction (7) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Ser for the 77<sup>th</sup> Thr in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 16 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture



was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 7 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 7, in which it is known that the 77<sup>th</sup> Thr in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

[Table 7]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 7	77 <sup>th</sup> position	Thr	Ser	ACC	TCC

	in $\alpha$ - subunit				
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[Reference Example 8]

Construction (8) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Ala for the 90<sup>th</sup> Gly in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 17 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 8 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 8, in which it is known that the 90<sup>th</sup> Gly in  
25 the  $\alpha$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Ala.

[Table 8]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 8	90 <sup>th</sup> position in $\alpha$ -subunit	Gly	Ala	GGC	GCC

[Reference Example 9]

5 Construction (9) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ala for the 102<sup>nd</sup> Val in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 18 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 9 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 9, in which it is known that the 102<sup>nd</sup> Val in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

[Table 9]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 9	102 <sup>nd</sup> position in $\alpha$ -subunit	Val	Ala	GTC	GCC

[Reference Example 10]

Construction (10) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ile for the 106<sup>th</sup> Val in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
5 Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 19 in the Sequence Listing and 50 pmols of an M13 primer M4 (having  
10 the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15  
15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV  
20 (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of  
25 the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 10 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 10, in which it is known that the 106<sup>th</sup> Val in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ile.

[Table 10]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 10	106 <sup>th</sup> position	Val	Ile	GTC	ATC



	in $\alpha$ - subunit				
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[Reference Example 11]

Construction (11) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Tyr for the 126<sup>th</sup> Phe in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 20 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 11 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 11, in which it is known that the 126<sup>th</sup> Phe in  
25 the  $\alpha$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Tyr.

[Table 11]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 11	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

[Reference Example 12]

5 Construction (12) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Glu for the 130<sup>th</sup> Gln in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 21 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 12 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 12, in which it is known that the 130<sup>th</sup> Gln in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

[Table 12]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 12	130 <sup>th</sup> position in $\alpha$ -subunit	Gln	Glu	CAG	GAG

[Reference Example 13]

Construction (13) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Val for the 142<sup>nd</sup> Leu in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
5 Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 22 in the Sequence Listing and 50 pmols of an M13 primer M4 (having  
10 the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15  
15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV  
20 (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of  
25 the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 13 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 13, in which it is known that the 142<sup>nd</sup> Leu in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

[Table 13]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 13	142 <sup>nd</sup> position	Leu	Val	CTG	GTG

	in $\alpha$ - subunit				
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[Reference Example 14]

Construction (14) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Asp for the 146<sup>th</sup> Glu in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 23 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a



reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the

5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 14 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 14, in which it is known that the 146<sup>th</sup> Glu in  
25 the  $\alpha$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Asp.

[Table 14]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 14	146 <sup>th</sup> position in $\alpha$ -subunit	Glu	Asp	GAG	GAC

[Reference Example 15]

5 Construction (15) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Thr for the 187<sup>th</sup> Ala in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference  
10 Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 24 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 15 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 15, in which it is known that the 187<sup>th</sup> Ala in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

10 [Table 15]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 15	187 <sup>th</sup> position in $\alpha$ -subunit	Ala	Thr	GCC	ACC

[Reference Example 16]

Construction (16) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Leu for the 194<sup>th</sup> Ser in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 25 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 16 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 16, in which it is known that the 194<sup>th</sup> Ser in the  $\alpha$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

[Table 16]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 16	194 <sup>th</sup> position	Ser	Leu	TCG	TTG

	in $\alpha$ - subunit				
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[Reference Example 17]

Construction (17) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Glu for the 203<sup>rd</sup> Ala in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 26 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 17 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 17, in which it is known that the 203<sup>rd</sup> Ala in  
25 the  $\alpha$ -subunit of the nitrile hydratase from the clone shown



therein was substituted with Glu.

[Table 17]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 17	203 <sup>rd</sup> position in $\alpha$ -subunit	Ala	Glu	GCG	GAG

[Reference Example 18]

5 Construction (18) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Val for the 20<sup>th</sup> Ala in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 27 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 18 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 18, in which it is known that the 20<sup>th</sup> Ala in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

10 [Table 18]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 18	20 <sup>th</sup> position in $\beta$ -subunit	Ala	Val	GCG	GTG

[Reference Example 19]

Construction (19) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Asn for the 21<sup>st</sup> Asp in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 28 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 19 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 19, in which it is known that the 21<sup>st</sup> Asp in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Asn.

[Table 19]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 19	21 <sup>st</sup> position	Asp	Asn	GAC	AAC

	in $\beta$ - subunit				
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[Reference Example 20]

Construction (20) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Asp for the 108<sup>th</sup> Glu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 29 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 20 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 20, in which it is known that the 108<sup>th</sup> Glu in  
25 the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Asp.

[Table 20]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 20	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT

[Reference Example 21]

5 Construction (21) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Pro for the 108<sup>th</sup> Glu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 30 in the



Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 21 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 21, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Pro.

10 [Table 21]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 21	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Pro	GAG	CCG

[Reference Example 22]

Construction (22) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Ser for the 108<sup>th</sup> Glu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 31 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 22 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 22, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

[Table 22]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 22	108 <sup>th</sup> position	Glu	Ser	GAG	TCG

	in $\beta$ - subunit				
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[Reference Example 23]

Construction (23) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Arg for the 108<sup>th</sup> Glu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1..

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 32 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 23 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 23, in which it is known that the 108<sup>th</sup> Glu in  
25 the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Arg.

[Table 23]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 23	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Arg	GAG	CGG

[Reference Example 24]

5 Construction (24) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Cys for the 108<sup>th</sup> Glu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 33 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 24 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and



the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 24, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Cys.

10 [Table 24]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 24	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Cys	GAG	TGC

[Reference Example 25]

Construction (25) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Leu for the 108<sup>th</sup> Glu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
5 Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 34 in the Sequence Listing and 50 pmols of an M13 primer M4 (having  
10 the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15  
15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV  
20 (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of  
25 the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 25 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 25, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

[Table 25]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 25	108 <sup>th</sup> position	Glu	Leu	GAG	CTG

	in $\beta$ - subunit				
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[Reference Example 26]

Construction (26) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        For the substitution with Thr for the 108<sup>th</sup> Glu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 35 in the  
15        Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20        one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 26 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 26, in which it is known that the 108<sup>th</sup> Glu in  
25 the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Thr.

[Table 26]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 26	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Thr	GAG	ACG

[Reference Example 27]

5 Construction (27) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Asp for the 200<sup>th</sup> Ala in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 36 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 27 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 27, in which it is known that the 200<sup>th</sup> Ala in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

10 [Table 27]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 27	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Asp	GCC	GAC

[Reference Example 28]

Construction (28) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Ile for the 200<sup>th</sup> Ala in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,



the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 37 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 28 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 28, in which it is known that the 200<sup>th</sup> Ala in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ile.

[Table 28]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 28	200 <sup>th</sup> position	Ala	Ile	GCC	ATC

	in $\beta$ - subunit				
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[Reference Example 29]

Construction (29) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Val for the 200<sup>th</sup> Ala in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Reference  
Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Reference Example 1 as the template, PCRs of two different  
types were carried out. For the PCR reaction No. 1, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of the  
primer having the sequence as forth in SEQ ID No: 38 in the  
15   Sequence Listing and 50 pmols of an M13 primer M4 (having  
the sequence as forth in SEQ ID No: 8 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
20   one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 29 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 29, in which it is known that the 200<sup>th</sup> Ala in  
25 the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Val.

[Table 29]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 29	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Val	GCC	GTC

[Reference Example 30]

5 Construction (30) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Glu for the 200<sup>th</sup> Ala in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 39 in the

Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 30 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 30, in which it is known that the 200<sup>th</sup> Ala in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

10 [Table 30]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 30	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAG

[Reference Example 31]

Construction (31) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Tyr for the 212<sup>th</sup> Ser in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,

the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Reference Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Reference Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 40 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture



was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 31 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 31, in which it is known that the 212<sup>th</sup> Ser in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Tyr.

[Table 31]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 31	212 <sup>th</sup> position	Ser	Tyr	TCC	TAC

	in $\beta$ - subunit				
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[Reference Example 32]

Construction (32) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 5 (where the 19<sup>th</sup> Ala in  
the  $\alpha$ -subunit was substituted with Val) and that from the  
clone No. 11 (where the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was  
10 substituted with Tyr) still had the nitrile hydratase  
activity.

10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
Ampicillin was added to this medium to have a final  
15 concentration of 100  $\mu$ g/ml. On the medium, one platinum  
loop of the cells of the clone No. 11 as prepared in  
Reference Example 11 was inoculated and incubated therein at  
37°C for about 20 hours with stirring at 300 rpm. 1 ml of  
the resulting culture was put into a suitable centrifugal  
20 tube, and this was subjected to centrifugation (15,000 rpm,  
5 minutes) to separate the cells from the culture. The  
plasmid DNA of the clone No. 11 was prepared from the cells

by alkaline SDS extraction.

Using 1 µg of the plasmid DNA of the clone No. 11 as the template, PCR of two different types was carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 14 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was

carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 32 was then obtained in completely the same manner as in Reference Example 1.

5           Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

          Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10   nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 32, in which it is known that the 19<sup>th</sup> Ala in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted  
15   with Val and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

[Table 32]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 32	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG

	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
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[Reference Example 33]

Construction (33) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 1 (where the 6<sup>th</sup> Leu in  
the  $\alpha$ -subunit was substituted with Met) and that from the  
clone No. 32 (where the 19<sup>th</sup> Ala in the  $\alpha$ -subunit was  
10 substituted with Val and the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was  
substituted with Tyr) still had the nitrile hydratase  
activity.

10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
15 Ampicillin was added to this medium to have a final  
concentration of 100  $\mu$ g/ml. On the medium, one platinum  
loop of the cells of the clone No. 32 as prepared in  
Reference Example 32 was inoculated and incubated therein at  
37°C for about 20 hours with stirring at 300 rpm. 1 ml of  
20 the resulting culture was put into a suitable centrifugal  
tube, and this was subjected to centrifugation (15,000 rpm,  
5 minutes) to separate the cells from the culture. The

plasmid DNA of the clone No. 32 was prepared from the cells by alkaline SDS extraction.

Using 1 µg of the plasmid DNA of the clone No. 32 as the template, PCRs of two different types were carried out.

5 For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 7 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition  
10 of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the  
15 PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition  
20 of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel  
25 electrophoresis (where the agarose concentration was 1.0% by

weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 33 was then obtained in completely the same manner as in

5 Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
10 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 33, in which it is known that the 6<sup>th</sup> Leu in  
15 the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Met, the 19<sup>th</sup> Ala in the same was substituted with Val and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

[Table 33]

Clone Number	Mutated Site  (in $\alpha$ - subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant

No. 33	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Met	CTG	ATG
	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

[Reference Example 34]

Construction (34) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 2 (where the 6<sup>th</sup> Leu in  
the  $\alpha$ -subunit was substituted with Thr) and that from the  
clone No. 32 (where the 19<sup>th</sup> Ala in the  $\alpha$ -subunit was  
10 substituted with Val and the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was  
substituted with Tyr) still had the nitrile hydratase  
activity.

Using 1  $\mu$ g of the plasmid DNA of the clone No. 32 as  
prepared in Reference Example 33 as the template, PCRs of  
15 two different types were carried out. For the PCR reaction



No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 11 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the  
5 Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension  
10 (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the  
15 Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
20 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 34 was then obtained in  
25 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 34, in which it is known that the 6<sup>th</sup> Leu in  
10 the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Thr, the 19<sup>th</sup> Ala in the same was substituted with Val and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

[Table 34]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 34	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Thr	CTG	ACG
	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

[Reference Example 35]

Construction (35) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 3 (where the 6<sup>th</sup> Leu in  
the  $\alpha$ -subunit was substituted with Ala) and that from the  
clone No. 32 (where the 19<sup>th</sup> Ala in the  $\alpha$ -subunit was  
10 substituted with Val and the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was  
substituted with Tyr) still had the nitrile hydratase  
activity.

          Using 1  $\mu$ g of the plasmid DNA of the clone No. 32 as  
prepared in Reference Example 33 as the template, PCRs of  
15 two different types were carried out. For the PCR reaction  
No. 1, a reaction system of 50  $\mu$ l in total comprising 50  
pmols of the primer having the sequence as forth in SEQ ID  
No: 12 in the Sequence Listing and 50 pmols of an M13 primer  
M4 (having the sequence as forth in SEQ ID No: 8 in the  
20 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
25 (72°C) for 120 seconds. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
(having the sequence as forth in SEQ ID No: 10 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 35 was then obtained in  
15 completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 35, in which it is known that the 6<sup>th</sup> Leu in  
25 the  $\alpha$ -subunit in the wild nitrile hydratase was substituted

with Ala, the 19<sup>th</sup> Ala in the same was substituted with Val and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

[Table 35]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 35	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Ala	CTG	GCG
	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

5 [Reference Example 36]

Construction (36) of the Substituted Amino Acid Having Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 20 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Asp) and that from the

clone No. 31 (where the 212<sup>th</sup> Ser in the  $\beta$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test  
5 tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum loop of the cells of the clone No. 31 as prepared in Reference Example 31 was inoculated and incubated therein at  
10 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA of the clone No. 31 was prepared from the cells  
15 by alkaline SDS extraction.

Using 1  $\mu$ g of the plasmid DNA of the clone No. 31 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence  
20 as forth in SEQ ID No: 29 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25  
25 PCR cycles, in which one PCR cycle comprised thermal

denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence  
5 as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out  
10 following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was  
15 carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 36 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
20 determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain  
25 termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 36, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Asp and the 212<sup>th</sup> Ser in the same was substituted with

5 Tyr.

[Table 36]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 36	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

[Reference Example 37]

Construction (37) of the Substituted Amino Acid Having

10 Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 23 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Arg) and that from the



clone No. 31 (where the 212<sup>th</sup> Ser in the  $\beta$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

Using 1  $\mu$ g of the plasmid DNA of the clone No. 31 as  
5 prepared in Reference Example 36 as the template, PCRs of  
two different types were carried out. For the PCR reaction  
No. 1, a reaction system of 50  $\mu$ l in total comprising 50  
pmols of the primer having the sequence as forth in SEQ ID  
No: 32 in the Sequence Listing and 50 pmols of an M13 primer  
10 M4 (having the sequence as forth in SEQ ID No: 8 in the  
Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
15 seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 120 seconds. For the PCR reaction No. 2, a  
reaction system of 50  $\mu$ l in total comprising 50 pmols of an  
MUT4 primer (having the sequence as forth in SEQ ID No: 9 in  
the Sequence Listing) and 50 pmols of an M13 primer RV  
20 (having the sequence as forth in SEQ ID No: 10 in the  
Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
25 the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture

was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 37 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 37, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Arg and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 37]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant

No. 37	108 <sup>th</sup> position in $\beta$ - subunit	Glu	Arg	GAG	CGG
	212 <sup>th</sup> position in $\beta$ - subunit	Ser	Tyr	TCC	TAC

[Reference Example 38]

Construction (38) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 27 (where the 200<sup>th</sup> Ala  
in the  $\beta$ -subunit was substituted with Asp) and that from the  
clone No. 31 (where the 212<sup>th</sup> Ser in the  $\beta$ -subunit was  
10 substituted with Tyr) still had the nitrile hydratase  
activity.

          Using 1  $\mu$ g of the plasmid DNA of the clone No. 31 as  
prepared in Reference Example 36 as the template, PCRs of  
two different types were carried out. For the PCR reaction  
15 No. 1, a reaction system of 50  $\mu$ l in total comprising 50  
pmols of the primer having the sequence as forth in SEQ ID

No: 36 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
5 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an  
10 MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of  
20 the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 38 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were  
25 determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 38, in which it is known that the 200<sup>th</sup> Ala in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Asp and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 38]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 38	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Asp	GCC	GAC
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

[Reference Example 39]

Construction (39) of the Substituted Amino Acid Having Nitrile Hydratase Activity

This is to demonstrate that the mutant with the

substituted amino acid sequence comprising both of the mutated position from the clone No. 30 (where the 200<sup>th</sup> Ala in the  $\beta$ -subunit was substituted with Glu) and that from the clone No. 31 (where the 212<sup>th</sup> Ser in the  $\beta$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

Using 1  $\mu$ g of the plasmid DNA of the clone No. 31 as prepared in Reference Example 36 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 39 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds.. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 39 was then obtained in completely the same manner as in Reference Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 39, in which it is known that the 200<sup>th</sup> Ala in the β-subunit in the wild nitrile hydratase was substituted with Glu and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 39]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\beta$ -subunit)	Wild Type	Mutant	Wild Type	Mutant
No. 39	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAG
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

[Example 1]

Construction (40) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           For the substitution with Met for the 36<sup>th</sup> Thr in the  $\alpha$ -subunit, introduction of site-specific mutation was performed using a "LA PCR in vitro mutagenesis Kit" (manufactured by Takara Shuzo Co., Ltd.). Hereinafter, the "LA PCR in vitro mutagenesis Kit" is simply referred to as  
10 the kit. In following Examples, the kit was handled on the basis of the principle thereof and in accordance with the manufacturer's instructions for the kit.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes.  
15 Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum



loop of the cells of MT-10822 were inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation  
5 (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid pPT-DB1 was prepared from the cells by alkaline SDS extraction.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No.  
10 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 41 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the  
15 manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a  
20 reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. From each of these PCR reaction mixtures, the excess primers and dNTP were removed using Microcon 100 (manufactured by Takara Shuzo Co., Ltd.), and then TE was added to each of the mixtures to prepare 50  $\mu$ l each of TE solutions. An annealing solution of 47.5  $\mu$ l in total containing 0.5  $\mu$ l of both of the above TE solutions (for the composition of the system, the manufacturer's instructions for the kit were followed) was prepared, and this solution was subjected to annealing by performing thermal denaturation of the solution at 98°C for 10 minutes, subsequently cooling the solution to 37°C at a constant cooling rate over a period of 60 minutes, and then maintaining it at 37°C for 15 minutes. To this annealed solution, 0.5  $\mu$ l of TaKaRa LA Taq was added, and the solution was heated at 72°C for 3 minutes, thus completing the formation of heterologous double-stranded DNA. This was then subjected to PCR reaction No. 3. For the PCR reaction No. 3, a reaction system of 50  $\mu$ l in total comprising 50

pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. After completion of the PCR reaction 3, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, a product by Sigma Corporation; agarose concentration of 0.8% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product of about 2 kbp. Subsequently, an agarose fragment comprising only a DNA fragment of about 2 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was then subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10 µl of TE. The amplified DNA fragment of about 2 kbp thus purified was cleaved by means of restriction endonucleases EcoRI and HindIII, and then subjected to

phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10  $\mu$ l of TE. Likewise, pPT-DB1 was cleaved by means of EcoRI and HindIII, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, a product by Sigma Corporation; agarose concentration of 0.7%). An agarose fragment comprising only the DNA fragment of about 2.7 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of the TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10  $\mu$ l of TE. Thus obtained DNA fragments of about 2 kbp and of about 2.7 kbp were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.). A competent cell of *E. coli* HB101 (manufactured by Toyobo Co., Ltd.) was transformed with the reaction product to obtain a transformant No. 40.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the

nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 40, in which it is known that the 36<sup>th</sup> Thr in the  $\alpha$ -subunit of the nitrile hydratase from the clone was substituted with Met.

[Table 40]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 40	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	ACG	ATG

[Example 2]

10 Construction (41) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with His for the 71<sup>st</sup> Arg in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in

Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 42 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA

product. A transformant No. 41 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 41, in which it is known that the 71<sup>st</sup> Arg in the  $\alpha$ -subunit of the nitrile hydratase from the clone was substituted with His.

[Table 41]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 41	71 <sup>st</sup> position in $\alpha$ -subunit	Arg	His	CGT	CAT

15

[Example 3]

Construction (42) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

For the substitution with Asp for the 148<sup>th</sup> Gly in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
5 the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
10 of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 43 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
15 instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
20 system of 50  $\mu$ l in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was



used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the  
5 agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 42 was then obtained in completely the same manner as in Example 1.

10        Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

         Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
15 nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 42, in which it is known that the 148<sup>th</sup> Gly in the α-subunit of the nitrile hydratase from the clone was  
20 substituted with Asp.

[Table 42]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\alpha$ -subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 42	148 <sup>th</sup> position in $\alpha$ -subunit	Gly	Asp	GGC	GAC

[Example 4]

Construction (43) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           For the substitution with Arg for the 204<sup>th</sup> Val in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 44 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle

comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
5 primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
15 the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 43 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
20 determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
25 termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 43, in which it is known that the 204<sup>th</sup> Val in the  $\alpha$ -subunit of the nitrile hydratase from the clone was substituted with Arg.

5 [Table 43]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 43	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Arg	GTC	CGC

[Example 5]

Construction (44) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 For the substitution with Lys for the 204<sup>th</sup> Val in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
15 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system

of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 45 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 44 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
 5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 44, in which it is known that the 204<sup>th</sup> Val in  
 10 the  $\alpha$ -subunit of the nitrile hydratase from the clone was substituted with Lys.

[Table 44]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 44	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Lys	GTC	AAA

[Example 6]

15 Construction (45) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Trp for the 204<sup>th</sup> Val in the  $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

5        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 46 in the Sequence  
10    Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
15    comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the  
20    Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
25    procedure as the PCR reaction No. 1. After completion of

the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 45 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 45, in which it is known that the 204<sup>th</sup> Val in the  $\alpha$ -subunit of the nitrile hydratase from the clone was substituted with Trp.

[Table 45]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 45	204 <sup>th</sup>	Val	Trp	GTC	TGG



	position in $\alpha$ - subunit				
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[Example 7]

Construction (46) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Thr for the 204<sup>th</sup> Val in the  
 $\alpha$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 47 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction

system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 46 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 46, in which it is known that the 204<sup>th</sup> Val in the α-subunit of the nitrile hydratase from the clone was

substituted with Thr.

[Table 46]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 46	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Thr	GTC	ACC

[Example 8]

5 Construction (47) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Asp for the 10<sup>th</sup> Thr in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having  
15 the sequence as forth in SEQ ID No: 48 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the

sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
5 comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
10 Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
20 being possible to confirm the presence of an amplified DNA  
product. A transformant No. 47 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
25 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

5 Autosequencer 373A manufactured by ABI. The results are shown in Table 47, in which it is known that the 10<sup>th</sup> Thr in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

[Table 47]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 47	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Asp	ACC	GAC

10

[Example 9]

Construction (48) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Glu for the 10<sup>th</sup> Thr in the  
15  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of

site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system  
5 of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 49 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's  
10 instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
15 system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
20 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the  
25 agarose concentration was 1.0% by weight), and an assay of

the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 48 was then obtained in completely the same manner as in Example 1.

5           Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

          Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10   nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 48, in which it is known that the 10<sup>th</sup> Thr in the  $\beta$ -subunit of the nitrile hydratase from the clone shown  
15   therein was substituted with Glu.

[Table 48]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 48	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Glu	ACC	GAA

[Example 10]

Construction (49) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Trp for the 10<sup>th</sup> Thr in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 50 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
25   the sequence as forth in SEQ ID No: 10 in the Sequence



Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of  
5 the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA  
10 product. A transformant No. 49 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

15 Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are  
20 shown in Table 49, in which it is known that the 10<sup>th</sup> Thr in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Trp.

[Table 49]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 49	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Trp	ACC	TGG

[Example 11]

Construction (50) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5 For the substitution with Gly for the 10<sup>th</sup> Thr in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

10 Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 51 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's

instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 50 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the

nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 50, in which it is known that the 10<sup>th</sup> Thr in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Gly.

[Table 50]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 50	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Gly	ACC	GGC

[Example 12]

10 Construction (51) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Tyr for the 10<sup>th</sup> Thr in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in

Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 52 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA

product. A transformant No. 51 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 51, in which it is known that the 10<sup>th</sup> Thr in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Tyr.

[Table 51]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 51	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Tyr	ACC	TAC

Construction (52) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

For the substitution with Cys for the 10<sup>th</sup> Thr in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
5 the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
10 of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 53 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
15 instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
20 system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 52 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 52, in which it is known that the 10<sup>th</sup> Thr in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Cys.

[Table 52]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\beta$ -subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 52	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Cys	ACC	TGC

[Example 14]

Construction (53) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           For the substitution with Gly for the 32<sup>nd</sup> Val in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

10           Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 54 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
15           sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle

comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
5 primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
15 the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 53 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
20 determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
25 termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 53, in which it is known that the 32<sup>nd</sup> Val in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Gly.

5 [Table 53]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 53	32 <sup>nd</sup> position in $\beta$ -subunit	Val	Gly	GTC	GGC

[Example 15]

Construction (54) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 For the substitution with Thr for the 37<sup>th</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

15 Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system

of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 55 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 54 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
 5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 54, in which it is known that the 37<sup>th</sup> Phe in  
 10 the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

[Table 54]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 54	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Thr	TTC	ACC

[Example 16]

15 Construction (55) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ala for the 37<sup>th</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

5        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 56 in the Sequence  
10    Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
15    comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the  
20    Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
25    procedure as the PCR reaction No. 1. After completion of

the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 55 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 55, in which it is known that the 37<sup>th</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

[Table 55]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 55	37 <sup>th</sup>	Phe	Ala	TTC	GCC

	position in $\beta$ - subunit				
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[Example 17]

Construction (56) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Leu for the 37<sup>th</sup> Phe in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 57 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction



system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence

5 Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
10 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 56 was then obtained in  
15 completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
20 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 56, in which it is known that the 37<sup>th</sup> Phe in  
25 the β-subunit of the nitrile hydratase from the clone shown

therein was substituted with Leu.

[Table 56]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 56	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Leu	TTC	CTC

[Example 18]

5 Construction (57) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ile for the 37<sup>th</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having  
15 the sequence as forth in SEQ ID No: 58 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the

sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
5 comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
10 Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
20 being possible to confirm the presence of an amplified DNA  
product. A transformant No. 57 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
25 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

5 Autosequencer 373A manufactured by ABI. The results are shown in Table 57, in which it is known that the 37<sup>th</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ile.

[Table 57]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 57	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Ile	TTC	ATC

10

[Example 19]

Construction (58) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Val for the 37<sup>th</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of

site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system  
5 of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 59 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's  
10 instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
15 system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
20 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the  
25 agarose concentration was 1.0% by weight), and an assay of

the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 58 was then obtained in completely the same manner as in Example 1.

5           Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

          Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10   nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 58, in which it is known that the 37<sup>th</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown  
15   therein was substituted with Val.

[Table 58]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 58	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC

[Example 20]

Construction (59) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        For the substitution with Glu for the 41<sup>st</sup> Phe in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

      Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10    Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 60 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15    sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20    annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
25    the sequence as forth in SEQ ID No: 10 in the Sequence

Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 59 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 59, in which it is known that the 41<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

[Table 59]



Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 59	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Glu	TTC	GAA

[Example 21]

Construction (60) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5 For the substitution with Thr for the 41<sup>st</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

10 Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 61 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's

instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 60 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the

nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 60, in which it is known that the 41<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

[Table 60]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 60	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Thr	TTC	ACC

[Example 22]

10 Construction (61) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ala for the 41<sup>st</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in

Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 62 in the Sequence

5 Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
10 comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the  
15 Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
20 procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it  
25 being possible to confirm the presence of an amplified DNA

product. A transformant No. 61 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 61, in which it is known that the 41<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

[Table 61]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 61	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Ala	TTC	GCC

## Construction (62) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Leu for the 41<sup>st</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,

5. the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system  
10 of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 63 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's  
15 instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
20 system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 62 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 62, in which it is known that the 41<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

[Table 62]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\beta$ - subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 62	41 <sup>st</sup> position in $\beta$ - subunit	Phe	Leu	TTC	CTC

[Example 24]

Construction (63) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Ile for the 41<sup>st</sup> Phe in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10 Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 64 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle



comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
5 primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
15 the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 63 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
20 determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
25 termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 63, in which it is known that the 41<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ile.

5 [Table 63]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 63	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Ile	TTC	ATC

[Example 25]

Construction (64) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 For the substitution with Val for the 41<sup>st</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
15 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system

of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 65 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) 5 (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) 10 for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence 15 Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture 20 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 64 was then obtained in 25 completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 64, in which it is known that the 41<sup>st</sup> Phe in  
10 the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

[Table 64]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 64	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC

[Example 26]

15 Construction (65) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Gly for the 46<sup>th</sup> Met in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

5        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 66 in the Sequence  
10    Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
15    comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4  
20    primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
25    procedure as the PCR reaction No. 1. After completion of

the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it  
5 being possible to confirm the presence of an amplified DNA product. A transformant No. 65 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and  
10 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an  
15 Autosequencer 373A manufactured by ABI. The results are shown in Table 65, in which it is known that the 46<sup>th</sup> Met in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Gly.

[Table 65]

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 65	46 <sup>th</sup>	Met	Gly	ATG	GGG

	position in $\beta$ - subunit				
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[Example 27]

Construction (66) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Tyr for the 46<sup>th</sup> Met in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 67 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction

system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 66 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 66, in which it is known that the 46<sup>th</sup> Met in the  $\beta$ -subunit of the nitrile hydratase from the clone shown



therein was substituted with Tyr.

[Table 66]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 66	46 <sup>th</sup> position in $\beta$ -subunit	Met	Tyr	ATG	TAT

[Example 28]

5 Construction (67) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Leu for the 46<sup>th</sup> Met in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having  
15 the sequence as forth in SEQ ID No: 68 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the

sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
5 comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
10 Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
20 being possible to confirm the presence of an amplified DNA  
product. A transformant No. 67 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
25 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

5 Autosequencer 373A manufactured by ABI. The results are shown in Table 67, in which it is known that the 46<sup>th</sup> Met in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

[Table 67]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 67	46 <sup>th</sup> position in $\beta$ -subunit	Met	Leu	ATG	CTG

10

[Example 29]

Construction (68) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Lys for the 46<sup>th</sup> Met in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of

site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1, as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system  
5 of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 69 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's  
10 instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
15 system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
20 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the  
25 agarose concentration was 1.0% by weight), and an assay of

the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 68 was then obtained in completely the same manner as in Example 1.

5           Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

          Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10   nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 68, in which it is known that the 46<sup>th</sup> Met in the  $\beta$ -subunit of the nitrile hydratase from the clone shown  
15   therein was substituted with Lys.

[Table 68]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 68	46 <sup>th</sup> position in $\beta$ -subunit	Met	Lys	ATG	AAG

[Example 30]

Construction (69) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        For the substitution with Asp for the 46<sup>th</sup> Met in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

      Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10    Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 70 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15    sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20    annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
25    the sequence as forth in SEQ ID No: 10 in the Sequence

Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 69 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 69, in which it is known that the 46<sup>th</sup> Met in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

[Table 69]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 69	46 <sup>th</sup> position in $\beta$ -subunit	Met	Asp	ATG	GAT

[Example 31]

Construction (70) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5 For the substitution with Gly for the 48<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
 10 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 71 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
 15 sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's



instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 70 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the

nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 70, in which it is known that the 48<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Gly.

[Table 70]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 70	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Gly	CTG	GGG

[Example 32]

10 Construction (71) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ala for the 48<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

15 Using 10 ng of the plasmid DNA pPT-DB1 as prepared in

Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 72 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA

product. A transformant No. 71 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 71, in which it is known that the 48<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

[Table 71]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 71	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Ala	CTG	GCG

15

[Example 33]

Construction (72) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

For the substitution with Val for the 48<sup>th</sup> Leu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
5 the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
10 of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 73 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
15 instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
20 system of 50  $\mu$ l in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 72 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 72, in which it is known that the 48<sup>th</sup> Leu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

[Table 72]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\beta$ - subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 72	48 <sup>th</sup> position in $\beta$ - subunit	Leu	Val	CTG	GTG

[Example 34]

Construction (73) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        For the substitution with Ser for the 48<sup>th</sup> Leu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

10        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 74 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15        sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle

comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
5 primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
15 the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 73 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
20 determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
25 termination method using a sequencing kit and an



Autosequencer 373A manufactured by ABI. The results are shown in Table 73, in which it is known that the 48<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

5 [Table 73]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 73	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Ser	CTG	TCG

[Example 35]

Construction (74) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 For the substitution with Thr for the 48<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
15 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system

of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 75 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) 5 (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) 10 for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence 15 Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture 20 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 74 was then obtained in 25 completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 74, in which it is known that the 48<sup>th</sup> Leu in  
10 the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Thr.

[Table 74]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 74	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Thr	CTG	ACG

[Example 36]

15 Construction (75) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Arg for the 48<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

5        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 76 in the Sequence  
10   Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
15   comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the  
20   Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
25   procedure as the PCR reaction No. 1. After completion of

the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 75 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 75, in which it is known that the 48<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Arg.

[Table 75]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 75	48 <sup>th</sup>	Leu	Arg	CTG	CGG

	position in $\beta$ - subunit				
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[Example 37]

Construction (76) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Ala for the 51<sup>st</sup> Phe in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 77 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction

system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 76 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 76; in which it is known that the 51<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Ala.

[Table 76]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 76	51 <sup>st</sup> position in $\beta$ -subunit	Phe	Ala	TTC	GCC

[Example 38]

5 Construction (77) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Val for the 51<sup>st</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having  
15 the sequence as forth in SEQ ID No: 78 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the



sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
5 comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
10 Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
20 being possible to confirm the presence of an amplified DNA  
product. A transformant No. 77 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
25 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

5 Autosequencer 373A manufactured by ABI. The results are shown in Table 77, in which it is known that the 51<sup>st</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

[Table 77]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 77	51 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC

10

[Example 39]

Construction (78) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Phe for the 72<sup>nd</sup> Trp in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of

site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system  
5 of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 79 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's  
10 instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
15 system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
20 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the  
25 agarose concentration was 1.0% by weight), and an assay of

the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 78 was then obtained in completely the same manner as in Example 1.

5           Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

          Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10   nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 78, in which it is known that the 72<sup>nd</sup> Trp in the  $\beta$ -subunit of the nitrile hydratase from the clone shown  
15   therein was substituted with Phe.

[Table 78]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 78	72 <sup>nd</sup> position in $\beta$ -subunit	Trp	Phe	TGG	TTT

[Example 40]

Construction (79) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        For the substitution with Ala for the 118<sup>th</sup> Phe in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

      Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10 Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 80 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20 annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
25 the sequence as forth in SEQ ID No: 10 in the Sequence

Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 79 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 79, in which it is known that the 118<sup>th</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

[Table 79]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 79	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Ala	TTC	GCC

[Example 41]

Construction (80) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5 For the substitution with Leu for the 118<sup>th</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

10 Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 81 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's

instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 80 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the



nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 80, in which it is known that the 118<sup>th</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

[Table 80]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 80	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Leu	TTC	CTC

[Example 42]

10 Construction (81) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Ile for the 118<sup>th</sup> Phe in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in

Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 82 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA

product. A transformant No. 81 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and  
5 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an  
10 Autosequencer 373A manufactured by ABI. The results are shown in Table 81, in which it is known that the 118<sup>th</sup> Phe in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ile.

[Table 81]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 81	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Ile	TTC	ATC

## Construction (82) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Val for the 118<sup>th</sup> Phe in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
5 the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
10 of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 83 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
15 instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
20 system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 82 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 82, in which it is known that the 118<sup>th</sup> Phe in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Val.

[Table 82]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\beta$ - subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 82	118 <sup>th</sup> position in $\beta$ - subunit	Phe	Val	TTC	GTC

[Example 44]

Construction (83) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Ala for the 127<sup>th</sup> Leu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10 Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 84 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle

comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 83 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 83, in which it is known that the 127<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ala.

5 [Table 83]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 83	127 <sup>th</sup> position in $\beta$ -subunit	Leu	Ala	CTG	GCG

[Example 45]

Construction (84) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 For the substitution with Val for the 127<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
15 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system



of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 85 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 84 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
5 alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
Autosequencer 373A manufactured by ABI. The results are  
shown in Table 84, in which it is known that the 127<sup>th</sup> Leu in  
10 the  $\beta$ -subunit of the nitrile hydratase from the clone shown  
therein was substituted with Val.

[Table 84]

Clone Number	Mutated Site (in $\beta$ - subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 84	127 <sup>th</sup> position in $\beta$ - subunit	Leu	Val	CTG	GTG

[Example 46]

15 Construction (85) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

For the substitution with Ser for the 127<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

5        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 86 in the Sequence  
10   Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
15   comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the  
20   Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
25   procedure as the PCR reaction No. 1. After completion of

the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 85 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 85, in which it is known that the 127<sup>th</sup> Leu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

[Table 85]

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 85	127 <sup>th</sup>	Leu	Ser	CTG	TCG

	position in $\beta$ - subunit				
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[Example 47]

Construction (86) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        For the substitution with Gly for the 146<sup>th</sup> Arg in the  
          $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
         the plasmid DNA pPT-DB1 was subjected to introduction of  
         site-specific mutation in the same manner as in Example 1.

         Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10      Example 1 as the template, PCRs of two different types were  
         carried out. For the PCR reaction No. 1, a reaction system  
         of 50  $\mu$ l in total comprising 50 pmols of the primer having  
         the sequence as forth in SEQ ID No: 87 in the Sequence  
         Listing and 50 pmols of an M13 primer M4 (having the  
15      sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
         (for the composition of the system, the manufacturer's  
         instructions for the kit were followed) was used, and the  
         reaction consisted of 25 PCR cycles, in which one PCR cycle  
         comprised thermal denaturation (98°C) for 15 seconds,  
20      annealing (55°C) for 30 seconds and chain extension (72°C)  
         for 120 seconds. For the PCR reaction No. 2, a reaction

system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 86 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 86, in which it is known that the 146<sup>th</sup> Arg in the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Gly.

[Table 86]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 86	146 <sup>th</sup> position in $\beta$ -subunit	Arg	Gly	CGG	GGG

[Example 48]

5 Construction (87) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Leu for the 160<sup>th</sup> Arg in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of  
10 site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having  
15 the sequence as forth in SEQ ID No. 88 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the

sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
5 comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
10 Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
the DNA amplification product was carried out, thereby it  
20 being possible to confirm the presence of an amplified DNA  
product. A transformant No. 87 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
25 the conversion and the selectivity were 100%, respectively.



Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

5 Autosequencer 373A manufactured by ABI. The results are shown in Table 87, in which it is known that the 160<sup>th</sup> Arg in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Leu.

[Table 87]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 87	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Leu	CGG	CTG

10

[Example 49]

Construction (88) of the Substituted Amino Acid Having Nitrile Hydratase Activity

15 For the substitution with Trp for the 160<sup>th</sup> Arg in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of

site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system  
5 of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 89 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's  
10 instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
15 system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the  
20 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the  
25 agarose concentration was 1.0% by weight), and an assay of

the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 88 was then obtained in completely the same manner as in Example 1.

5           Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

          Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10   nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 88, in which it is known that the 160<sup>th</sup> Arg in the  $\beta$ -subunit of the nitrile hydratase from the clone shown  
15   therein was substituted with Trp.

[Table 88]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 88	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Trp	CGG	TGG

[Example 50]

Construction (89) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Glu for the 186<sup>th</sup> Leu in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 90 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
25   the sequence as forth in SEQ ID No: 10 in the Sequence

Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 89 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 89, in which it is known that the 186<sup>th</sup> Leu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Glu.

[Table 89]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 89	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Glu	CTG	GAG

[Example 51]

Construction (90) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5 For the substitution with Asp for the 186<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 91 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the  
15 sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's

instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 90 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the

nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 90, in which it is known that the 186<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Asp.

[Table 90]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 90	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Asp	CTG	GAT

[Example 52]

10 Construction (91) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Lys for the 186<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

15 Using 10 ng of the plasmid DNA pPT-DB1 as prepared in



Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 92 in the Sequence

5 Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
10 comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the  
15 Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
20 procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it  
25 being possible to confirm the presence of an amplified DNA

product. A transformant No. 91 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 91, in which it is known that the 186<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Lys.

[Table 91]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 91	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Lys	CTG	AAG

15

[Example 53]

Construction (92) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

For the substitution with Arg for the 186<sup>th</sup> Leu in the  
β-subunit, using the plasmid DNA pPT-DB1 as the template,  
5 the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
10 of 50 μl in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 93 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
15 instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
20 system of 50 μl in total comprising 50 pmols of an MUT4  
primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
25 manufacturer's instructions for the kit were followed) was

used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 92 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 92, in which it is known that the 186<sup>th</sup> Leu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Arg.

[Table 92]

Clone Number	Mutated Site	Change in Amino Acid Sequence	Change in Base Sequence
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	(in $\beta$ - subunit)	Before Substitution	After Substitution	Before Substitution	After Substitution
No. 92	186 <sup>th</sup> position in $\beta$ - subunit	Leu	Arg	CTG	CGG

[Example 54]

Construction (93) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Asn for the 186<sup>th</sup> Leu in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 94 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing).  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle

comprised thermal denaturation (98°C) for 15 seconds,  
annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction  
system of 50 µl in total comprising 50 pmols of an MUT4  
5 primer (having the sequence as forth in SEQ ID No: 9 in the  
Sequence Listing) and 50 pmols of an M13 primer RV (having  
the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1. After completion of  
the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture  
was subjected to agarose gel electrophoresis (where the  
agarose concentration was 1.0% by weight), and an assay of  
15 the DNA amplification product was carried out, thereby it  
being possible to confirm the presence of an amplified DNA  
product. A transformant No. 93 was then obtained in  
completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were  
20 determined in the same manner as in Reference Example 1, and  
the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
25 termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 93, in which it is known that the 186<sup>th</sup> Leu in the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Asn.

5 [Table 93]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 93	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Asn	CTG	AAC

[Example 55]

Construction (94) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 For the substitution with Ser for the 186<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
15 Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system

of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 95 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) 5 (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) 10 for 120 seconds. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence 15 Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture 20 was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 94 was then obtained in 25 completely the same manner as in Example 1.



Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
 5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 94, in which it is known that the 186<sup>th</sup> Leu in  
 10 the  $\beta$ -subunit of the nitrile hydratase from the clone shown therein was substituted with Ser.

[Table 94]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 94	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Ser	CTG	TCG

[Example 56]

15 Construction (95) of the Substituted Amino Acid Having Nitrile Hydratase Activity

For the substitution with Gly for the 186<sup>th</sup> Leu in the  $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template, the plasmid DNA pPT-DB1 was subjected to introduction of site-specific mutation in the same manner as in Example 1.

5        Using 10 ng of the plasmid DNA pPT-DB1 as prepared in Example 1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 96 in the Sequence  
10   Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle  
15   comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction  
20   system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence  
Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
25   procedure as the PCR reaction No. 1. After completion of

the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 95 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 95, in which it is known that the 186<sup>th</sup> Leu in the β-subunit of the nitrile hydratase from the clone shown therein was substituted with Gly.

[Table 95]

Clone Number	Mutated Site (in β-subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 95	186 <sup>th</sup>	Leu	Gly	CTG	GGG

	position in $\beta$ - subunit				
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[Example 57]

Construction (96) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           For the substitution with Gly for the 217<sup>th</sup> Asp in the  
 $\beta$ -subunit, using the plasmid DNA pPT-DB1 as the template,  
the plasmid DNA pPT-DB1 was subjected to introduction of  
site-specific mutation in the same manner as in Example 1.

          Using 10 ng of the plasmid DNA pPT-DB1 as prepared in  
10   Example 1 as the template, PCRs of two different types were  
carried out. For the PCR reaction No. 1, a reaction system  
of 50  $\mu$ l in total comprising 50 pmols of the primer having  
the sequence as forth in SEQ ID No: 97 in the Sequence  
Listing and 50 pmols of an M13 primer M4 (having the  
15   sequence as forth in SEQ ID No: 8 in the Sequence Listing)  
(for the composition of the system, the manufacturer's  
instructions for the kit were followed) was used, and the  
reaction consisted of 25 PCR cycles, in which one PCR cycle  
comprised thermal denaturation (98°C) for 15 seconds,  
20   annealing (55°C) for 30 seconds and chain extension (72°C)  
for 120 seconds. For the PCR reaction No. 2, a reaction

system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 96 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 96, in which it is known that the 217<sup>th</sup> Asp in the  $\beta$ -subunit of the nitrile hydratase from the clone shown

therein was substituted with Gly.

[Table 96]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Before Substitution	After Substitution	Before Substitution	After Substitution
No. 96	217 <sup>th</sup> position in $\beta$ -subunit	Asp	Gly	GAC	GGC

[Example 58]

5 Construction (97) of the Substituted Amino Acid Having Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 40 (where the 36<sup>th</sup> Thr in  
10 the  $\alpha$ -subunit was substituted with Met) and that from the clone No. 11 (where the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test  
15 tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final

concentration of 100 µg/ml. On the medium, one platinum  
loop of the cells of the clone No. 11 as prepared in  
Reference Example 11 was inoculated and incubated therein at  
37°C for about 20 hours with stirring at 300 rpm. 1 ml of  
5 the resulting culture was put into a suitable centrifugal  
tube, and this was subjected to centrifugation (15,000 rpm,  
5 minutes) to separate the cells from the culture. The  
plasmid DNA was prepared from the cells by alkaline SDS  
extraction.

10       Using 10 ng of the prepared plasmid DNA as the template,  
PCRs of two different types were carried out. For the PCR  
reaction No. 1, a reaction system of 50 µl in total  
comprising 50 pmols of the primer having the sequence as  
forth in SEQ ID No: 41 in the Sequence Listing and 50 pmols  
15 of an M13 primer M4 (having the sequence as forth in SEQ ID  
No: 8 in the Sequence Listing) (for the composition of the  
system, the manufacturer's instructions for the kit were  
followed) was used, and the reaction consisted of 25 PCR  
cycles, in which one PCR cycle comprised thermal  
20 denaturation (98°C) for 15 seconds, annealing (55°C) for 30  
seconds and chain extension (72°C) for 120 seconds. For the  
PCR reaction No. 2, a reaction system of 50 µl in total  
comprising 50 pmols of an MUT4 primer (having the sequence  
as forth in SEQ ID No: 9 in the Sequence Listing) and 50  
25 pmols of an M13 primer RV (having the sequence as forth in

SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

5 After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the  
10 presence of an amplified DNA product. A transformant No. 97 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and  
15 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an  
20 Autosequencer 373A manufactured by ABI. The results are shown in Table 97, in which it is known that the 36<sup>th</sup> Thr in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Met and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

25 [Table 97]



Clone Number	Mutated Site (in $\alpha$ - subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 97	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	ACG	ATG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

[Example 59]

Construction (98) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 42 (where the 148<sup>th</sup> Gly  
in the  $\alpha$ -subunit was substituted with Asp) and that from the  
clone No. 43 (where the 204<sup>th</sup> Val in the  $\alpha$ -subunit was  
10 substituted with Arg) still had the nitrile hydratase  
activity.

10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
Ampicillin was added to this medium to have a final

concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 43 as prepared in Example 4 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, 10 PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 43 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition

of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 98 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 98, in which it is known that the 14<sup>th</sup> Gly in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Asp and the 204<sup>th</sup> Val in the same was substituted with Arg.

[Table 98]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 98	148 <sup>th</sup> position in $\alpha$ -subunit	Gly	Asp	GGC	GAC
	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Arg	GTC	CGC

[Example 60]

Construction (99) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 77 (where the 51<sup>st</sup> Phe in the  $\beta$ -subunit was substituted with Val) and that from the clone No. 20 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Asp) still had the nitrile hydratase activity.

10           10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final

concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 20 as prepared in Reference Example 20 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

10        Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 78 in the Sequence Listing and 50 pmols  
15        of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal  
20        denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50  
25        pmols of an M13 primer RV (having the sequence as forth in

SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

5 After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the  
10 presence of an amplified DNA product. A transformant No. 99 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and  
15 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an  
20 Autosequencer 373A manufactured by ABI. The results are shown in Table 99, in which it is known that the 51<sup>st</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Val and the 108<sup>th</sup> Glu in the same was substituted with Asp.

25 [Table 99]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 99	51 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT

[Reference Example 40]

Construction (100) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5 This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 20 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Asp) and that from the clone No. 30 (where the 200<sup>th</sup> Ala in the  $\beta$ -subunit was substituted with Glu) still had the nitrile hydratase activity.

10 10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final  
15 concentration of 100  $\mu$ g/ml. On the medium, one platinum

loop of the cells of the clone No. 30 as prepared in Reference Example 30 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, 10 PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 29 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID 15 No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 20 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in 25 SEQ ID No: 10 in the Sequence Listing) (for the composition



of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of

5 the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No.  
10 100 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

15 Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are  
20 shown in Table 100, in which it is known that the 108<sup>th</sup> Glu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Asp and the 200<sup>th</sup> Ala in the same was substituted with Glu.

[Table 100]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 100	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

[Example 61]

Construction (101) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 82 (where the 118<sup>th</sup> Phe in the  $\beta$ -subunit was substituted with Val) and that from the clone No. 30 (where the 200<sup>th</sup> Ala in the  $\beta$ -subunit was substituted with Glu) still had the nitrile hydratase activity.

10           10 ml of a liquid LB medium was put into a 30 ml test

tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 30 as prepared in

5 Reference Example 30 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The  
10 plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total  
15 comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 83 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were  
20 followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total  
25 comprising 50 pmols of an MUT4 primer (having the sequence

as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 101 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 101, in which it is known that the 118<sup>th</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Val and the 200<sup>th</sup> Ala in the same was

substituted with Glu.

[Table 101]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 101	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

[Example 62]

5 Construction (102) of the Substituted Amino Acid Having Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 88 (where the 160<sup>th</sup> Arg in the  $\beta$ -subunit was substituted with Trp) and that from the clone No. 92 (where the 186<sup>th</sup> Leu in the  $\beta$ -subunit was substituted with Arg) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final

concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 92 as prepared in Example 53 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, 10 PCR of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 89 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition

of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of

5 the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No.  
10 102 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

15 Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are  
20 shown in Table 102, in which it is known that the 160<sup>th</sup> Arg in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Trp and the 186<sup>th</sup> Leu in the same was substituted with Arg.

[Table 102]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 102	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Trp	CGG	TGG
	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Arg	CTG	CGG

[Example 63]

Construction (103) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 2 (where the 6<sup>th</sup> Leu in the  $\alpha$ -subunit was substituted with Thr) and that from the clone No. 97 (where the 36<sup>th</sup> Thr in the  $\alpha$ -subunit was substituted with Met and the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10           10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes.

15           Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum



loop of the cells of the clone No. 97 as prepared in Example 58 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this  
5 was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR  
10 reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 11 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the  
15 system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the  
20 PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition  
25 of the system, the manufacturer's instructions for the kit

were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of the reaction mixture was subjected to agarose gel

5 electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 103 was then obtained in completely the same manner as in  
10 Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
15 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 103, in which it is known that the 6<sup>th</sup> Leu in  
20 the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Thr, the 36<sup>th</sup> Thr in the same was substituted with Met and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

[Table 103]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 103	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Thr	CTG	ACG
	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	ACG	ATG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

[Example 64]

Construction (104) of the Substituted Amino Acid Having Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 41 (where the 71<sup>st</sup> Arg in the  $\alpha$ -subunit was substituted with His) and that from the clone No. 32 (where the 19<sup>th</sup> Ala in the  $\alpha$ -subunit was substituted with Val and the 126<sup>th</sup> Phe in the  $\alpha$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10           10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes.

Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 32 as prepared in Reference Example 32 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 42 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50

pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 104 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 104, in which it is known that the 19<sup>th</sup> Ala in the α-subunit in the wild nitrile hydratase was substituted with Val, the 71<sup>st</sup> Arg in the same was substituted with His and the 126<sup>th</sup> Phe in the same was substituted with Tyr.

[Table 104]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 104	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	71 <sup>st</sup> position in $\alpha$ -subunit	Arg	His	CGT	CAT
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC

[Example 65]

Construction (105) of the Substituted Amino Acid Having  
5 Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 40 (where the 36<sup>th</sup> Thr in the  $\alpha$ -subunit was substituted with Met) and that from the  
10 clone No. 98 (where the 148<sup>th</sup> Gly in the  $\alpha$ -subunit was substituted with Asp and the 204<sup>th</sup> Val in the  $\alpha$ -subunit was substituted with Arg) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test

tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 98 as prepared in Example 5 59 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was 10 prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as 15 forth in SEQ ID No: 41 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR 20 cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence 25 as forth in SEQ ID No: 9 in the Sequence Listing) and 50

pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 105 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 105, in which it is known that the 36<sup>th</sup> Thr in the α-subunit in the wild nitrile hydratase was substituted with Met, the 148<sup>th</sup> Gly in the same was substituted with Asp and the 204<sup>th</sup> Val in the same was substituted with Arg.



[Table 105]

Clone Number	Mutated Site (in $\alpha$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 105	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	ACG	ATG
	148 <sup>th</sup> position in $\alpha$ -subunit	Gly	Asp	GGC	GAC
	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Arg	GTC	CGC

[Example 66]

Construction (106) of the Substituted Amino Acid Having

5 Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 47 (where the 10<sup>th</sup> Thr in the  $\beta$ -subunit was substituted with Asp) and that from the  
10 clone No. 101 (where the 118<sup>th</sup> Phe in the  $\beta$ -subunit was substituted with Val and the 200th Ala in the  $\beta$ -subunit was substituted with Glu) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum  
5 loop of the cells of the clone No. 101 as prepared in Example 61 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5  
10 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR  
15 reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 48 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the  
20 system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the  
25 PCR reaction No. 2, a reaction system of 50 µl in total

comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 106 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 106, in which it is known that the 10<sup>th</sup> Thr in the β-subunit in the wild nitrile hydratase was substituted

with Asp, the 118<sup>th</sup> Phe in the same was substituted with Val and the 200<sup>th</sup> Ala in the same was substituted with Glu.

[Table 106]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 106	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Asp	ACC	GAC
	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

# 5 [Example 67]

Construction (107) of the Substituted Amino Acid Having Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 56 (where the 37<sup>th</sup> Phe in the  $\beta$ -subunit was substituted with Leu) and that from the clone No. 100 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Asp and the 200<sup>th</sup> Ala in the  $\beta$ -subunit was substituted with Glu) still had the nitrile hydratase

activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final  
5 concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 100 as prepared in Reference Example 40 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal  
10 tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template,  
15 PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 57 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID  
20 No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30  
25 seconds and chain extension (72°C) for 120 seconds. For the

PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in  
5 SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l of  
10 the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No.  
15 107 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

20 Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are  
25 shown in Table 107, in which it is known that the 37<sup>th</sup> Phe in

the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Leu, the 108<sup>th</sup> Glu in the same was substituted with Asp and the 200<sup>th</sup> Ala in the same was substituted with Glu.

[Table 107]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 107	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Leu	TTC	CTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

5

[Example 68]

Construction (108) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 58 (where the 37<sup>th</sup> Phe in the  $\beta$ -subunit was substituted with Val) and that from the clone No. 100 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Asp and the 200<sup>th</sup> Ala in the  $\beta$ -subunit was

substituted with Glu) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes.

5 Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 100 as prepared in Reference Example 40 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of  
10 the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

15 Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 59 in the Sequence Listing and 50 pmols  
20 of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal  
25 denaturation (98°C) for 15 seconds, annealing (55°C) for 30



seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50  
5 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.  
10 After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the  
15 presence of an amplified DNA product. A transformant No. 108 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and  
20 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an  
25 Autosequencer 373A manufactured by ABI. The results are

shown in Table 108, in which it is known that the 37<sup>th</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Val, the 108<sup>th</sup> Glu in the same was substituted with Asp and the 200<sup>th</sup> Ala in the same was substituted with Glu.

5 [Table 108]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 108	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

[Example 69]

Construction (109) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 63 (where the 41<sup>st</sup> Phe in the  $\beta$ -subunit was substituted with Ile) and that from the clone No. 99 (where the 51<sup>st</sup> Phe in the  $\beta$ -subunit was

substituted with Val and the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Asp) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum loop of the cells of the clone No. 99 as prepared in Example 60 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 64 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30

seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50

5 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

10 After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the

15 presence of an amplified DNA product. A transformant No. 109 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and

20 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

25 Autosequencer 373A manufactured by ABI. The results are

shown in Table 109, in which it is known that the 41<sup>st</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Ile, the 51<sup>st</sup> Phe in the same was substituted with Val and the 108<sup>th</sup> Glu in the same was substituted with Asp.

5 [Table 109]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 109	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Ile	TTC	ATC
	51 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT

[Example 70]

Construction (110) of the Substituted Amino Acid Having Nitrile Hydratase Activity

10 This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the

mutated position from the clone No. 68 (where the 46<sup>th</sup> Met in the  $\beta$ -subunit was substituted with Lys) and that from the clone No. 37 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Arg and the 212<sup>th</sup> Ser in the  $\beta$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum loop of the cells of the clone No. 37 as prepared in Reference Example 37 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 69 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the

system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1. After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 110 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by

alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are

5 shown in Table 110, in which it is known that the 46<sup>th</sup> Met in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Lys, the 108<sup>th</sup> Glu in the same was substituted with Arg and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 110]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 110	46 <sup>th</sup> position in $\beta$ -subunit	Met	Lys	ATG	AAG
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Arg	GAG	CGG
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

10

[Example 71]

Construction (111) of the Substituted Amino Acid Having Nitrile Hydratase Activity



This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 72 (where the 48<sup>th</sup> Leu in the  $\beta$ -subunit was substituted with Val) and that from the clone No. 37 (where the 108<sup>th</sup> Glu in the  $\beta$ -subunit was substituted with Arg and the 212<sup>th</sup> Ser in the  $\beta$ -subunit was substituted with Tyr) still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum loop of the cells of the clone No. 37 as prepared in Reference Example 37 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The plasmid DNA was prepared from the cells by alkaline SDS extraction.

Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmols of the primer having the sequence as forth in SEQ ID No: 73 in the Sequence Listing and 50 pmols

of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal

denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the

PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmols of an MUT4 primer (having the sequence

as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out

following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was

carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 111 was then obtained in completely the same manner as in Example 1.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and

the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 111, in which it is known that the 48<sup>th</sup> Leu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Val, the 108<sup>th</sup> Glu in the same was substituted with Arg and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 111]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 111	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Val	CTG	GTG
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Arg	GAG	CGG
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

[Example 72]

Construction (112) of the Substituted Amino Acid Having

### Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 85 (where the 127<sup>th</sup> Leu in the  $\beta$ -subunit was substituted with Ser) and that from the clone No. 102 (where the 160<sup>th</sup> Arg in the  $\beta$ -subunit was substituted with Trp and the 186<sup>th</sup> Leu in the  $\beta$ -subunit was substituted with Arg) still had the nitrile hydratase activity.

10        10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100  $\mu$ g/ml. On the medium, one platinum loop of the cells of the clone No. 102 as prepared in  
15        Example 62 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. The  
20        plasmid DNA was prepared from the cells by alkaline SDS extraction.

      Using 10 ng of the prepared plasmid DNA as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total  
25        comprising 50 pmols of the primer having the sequence as

forth in SEQ ID No: 86 in the Sequence Listing and 50 pmols of an M13 primer M4 (having the sequence as forth in SEQ ID No: 8 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were

5 followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 120 seconds. For the PCR reaction No. 2, a reaction system of 50 µl in total

10 comprising 50 pmols of an MUT4 primer (having the sequence as forth in SEQ ID No: 9 in the Sequence Listing) and 50 pmols of an M13 primer RV (having the sequence as forth in SEQ ID No: 10 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit

15 were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (where the agarose concentration was 1.0% by

20 weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product. A transformant No. 112 was then obtained in completely the same manner as in Example 1.

25       Thereafter, the addition rate and the selectivity were

determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 112, in which it is known that the 127<sup>th</sup> Leu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Ser, the 160<sup>th</sup> Arg in the same was substituted with Trp and the 186<sup>th</sup> Leu in the same was substituted with Arg.

[Table 112]

Clone Number	Mutated Site (in $\beta$ -subunit)	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 112	127 <sup>th</sup> position in $\beta$ -subunit	Leu	Ser	CTG	TCG
	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Trp	CGG	TGG
	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Arg	CTG	CGG

[Example 73]

Construction (113) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

This is to demonstrate that the mutant with the  
5 substituted amino acid sequence comprising both of the  
mutated position from the clone No. 34 and that from the  
clone No. 110 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
10 Ampicillin was added to this medium to have a final  
concentration of 100 µg/ml. On the medium, one platinum  
loop of the cells of the clone No. 34 as prepared in  
Reference Example 34 and the clone No. 110 as prepared in  
Example 70 was inoculated and incubated therein at 37°C for  
15 about 20 hours with stirring at 300 rpm. 1 ml of the  
resulting culture was put into a suitable centrifugal tube,  
and this was subjected to centrifugation (15,000 rpm, 5  
minutes) to separate the cells from the culture. From the  
cells extracted the plasmid DNA of the clone No. 34 and the  
20 plasmid DNA of the clone No. 110 through alkaline SDS  
extraction.

The plasmid DNA from the clone No. 110 was cleaved by  
means of restriction endonucleases EcoRI and NotI at the  
cleaving sites thereof, and then subjected to agarose gel  
25 electrophoresis (using Type VII low-melting-point agarose,

manufactured by Sigma Corporation; agarose concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 34 was cleaved by means of

5 restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of  
10 about 3.8 kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose  
15 melts were separately subjected to phenol/chloroform extraction and ethanol precipitation, to purify the DNA fragment. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the  
20 clone No. 110 and the DNA fragment of about 3.8 kbp obtained from the clone No. 34 were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced into competent cells of *E.coli* HB101 (manufactured by Toyobo  
25 Co.,Ltd.). Thus was obtained a transformant No. 113.



Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
5 alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 113, in which it is known that the 6<sup>th</sup> Leu in  
10 the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Thr, the 19<sup>th</sup> Ala in the same was substituted with Val, the 126<sup>th</sup> Phe in the same was substituted with Tyr, while the 46<sup>th</sup> Met in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Lys, the 108<sup>th</sup> Glu in the same was  
15 substituted with Arg, and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 113]

Clone Numbe r	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 113	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Thr	CTG	ACG

	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
	46 <sup>th</sup> position in $\beta$ -subunit	Met	Lys	ATG	AAG
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Arg	GAG	CGG
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

[Example 74]

Construction (114) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 34 and that from the  
clone No. 111 still had the nitrile hydratase activity.

10       10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
Ampicillin was added to this medium to have a final  
concentration of 100  $\mu$ g/ml. On the medium, one platinum  
loop of the cells of the clone No. 34 as prepared in  
Reference Example 34 and the clone No. 111 as prepared in

Example 71 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. From the cells extracted the plasmid DNA of the clone No. 34 and the plasmid DNA of the clone No. 111 through alkaline SDS extraction.

The plasmid DNA from the clone No. 111 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; agarose concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 34 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose

fragments were completely melted. The resulting agarose  
melts were separately subjected to phenol/chloroform  
extraction and ethanol precipitation, to purify the DNA  
fragments. Finally, these were separately dissolved in 10  
5  $\mu$ l of TE.

The DNA fragment of about 770 bp obtained from the  
clone No. 111 and the DNA fragment of about 3.8 kbp obtained  
from the clone No. 34 were subjected to DNA ligation, using  
a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.),  
10 to construct a plasmid. This plasmid DNA was introduced  
into competent cells of *E.coli* HB101 (manufactured by Toyobo  
Co.,Ltd.). Thus was obtained a transformant No. 114.

Thereafter, the addition rate and the selectivity were  
determined in the same manner as in Reference Example 1, and  
15 the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by  
alkaline SDS extraction. Next, the base sequence of the  
nitrile hydratase gene was determined by the dideoxy chain  
termination method using a sequencing kit and an  
20 Autosequencer 373A manufactured by ABI. The results are  
shown in Table 114, in which it is known that the 6<sup>th</sup> Leu in  
the  $\alpha$ -subunit in the wild nitrile hydratase was substituted  
with Thr, the 19<sup>th</sup> Ala in the same was substituted with Val,  
the 126<sup>th</sup> Phe in the same was substituted with Tyr, while the  
25 48<sup>th</sup> Leu in the  $\beta$ -subunit in the wild nitrile hydratase was

substituted with Val, the 108<sup>th</sup> Glu in the same was substituted with Arg, and the 212<sup>th</sup> Ser in the same was substituted with Tyr.

[Table 114]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 114	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Thr	CTG	ACG
	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Val	CTG	GTG
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Arg	GAG	CGG
	212 <sup>th</sup> position in $\beta$ -subunit	Ser	Tyr	TCC	TAC

5

[Example 75]

Construction (115) of the Substituted Amino Acid Having Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 35 and that from the clone No. 112 still had the nitrile hydratase activity.

5        10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 35 as prepared in  
10 Reference Example 35 and the clone No. 112 as prepared in Example 72 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5  
15 minutes) to separate the cells from the culture. From the cells extracted the plasmid DNA of the clone No. 35 and the plasmid DNA of the clone No. 112 through alkaline SDS extraction.

The plasmid DNA from the clone No. 112 was cleaved by  
20 means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 1.0%), through which the DNA fragment of  
25 about 770 bp was cut out of the agarose gel. In the same

manner, the plasmid DNA from the clone No. 35 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose melts were separately subjected to phenol/chloroform extraction and ethanol precipitation, to purify the DNA fragments. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 112 and the DNA fragment of about 3.8 kbp obtained from the clone No. 35 were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced into competent cells of *E.coli* HB101 (manufactured by Toyobo Co., Ltd.). Thus was obtained a transformant No. 115.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

5 Autosequencer 373A manufactured by ABI. The results are shown in Table 115, in which it is known that the 6<sup>th</sup> Leu in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Ala, the 19<sup>th</sup> Ala in the same was substituted with Val, the 126<sup>th</sup> Phe in the same was substituted with Tyr, while the  
10 127<sup>th</sup> Leu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Ser, the 160<sup>th</sup> Arg in the same was substituted with Trp, and the 186<sup>th</sup> Leu in the same was substituted with Arg.



[Table 115]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 115	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Ala	CTG	GCG
	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
	127 <sup>th</sup> position in $\beta$ -subunit	Leu	Ser	CTG	TCG
	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Trp	CGG	TGG
	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Arg	CTG	CGG

[Example 76]

Construction (116) of the Substituted Amino Acid Having  
5 Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 103 and that from the

clone No. 106 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final  
5 concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 103 as prepared in Example 63 and the clone No. 106 as prepared in Example 66 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting  
10 culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. From the cells extracted the plasmid DNA of the clone No. 103 and the plasmid DNA of the clone No. 106 through alkaline SDS  
15 extraction.

The plasmid DNA from the clone No. 106 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose,  
20 manufactured by Sigma Corporation; at an agarose concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 103 was cleaved by means of restriction endonucleases EcoRI and NotI at the  
25 cleaving sites thereof, and then subjected to agarose gel

electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the  
5 thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose melts were separately subjected to phenol/chloroform  
10 extraction and ethanol precipitation, to purify the DNA fragments. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 106 and the DNA fragment of about 3.8 kbp obtained  
15 from the clone No. 103 were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced into competent cells of *E.coli* HB101 (manufactured by Toyobo Co.,Ltd.). Thus was obtained a transformant No. 116.

20       Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
25 nitrile hydratase gene was determined by the dideoxy chain

termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 116, in which it is known that the 6<sup>th</sup> Leu in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Thr, the 36<sup>th</sup> Thr in the same was substituted with Met, the 126<sup>th</sup> Phe in the same was substituted with Tyr, while the 10<sup>th</sup> Thr in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Asp, the 118<sup>th</sup> Phe in the same was substituted with Val, and the 200<sup>th</sup> Ala in the same was substituted with Glu.

[Table 116]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 116	6 <sup>th</sup> position in $\alpha$ -subunit	Leu	Thr	CTG	ACG
	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	ACG	ATG
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Asp	ACC	GAC

	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

[Example 77]

Construction (117) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5        This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 104 and that from the  
clone No. 107 still had the nitrile hydratase activity.

10        10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
Ampicillin was added to this medium to have a final  
concentration of 100  $\mu$ g/ml. On the medium, one platinum  
loop of the cells of the clone No. 104 as prepared in  
Example 64 and the clone No. 107 as prepared in Example 67  
15        was inoculated and incubated therein at 37°C for about 20  
hours with stirring at 300 rpm. 1 ml of the resulting  
culture was put into a suitable centrifugal tube, and this  
was subjected to centrifugation (15,000 rpm, 5 minutes) to  
separate the cells from the culture. From the cells  
20        extracted the plasmid DNA of the clone No. 104 and the

plasmid DNA of the clone No. 107 through alkaline SDS extraction.

The plasmid DNA from the clone No. 107 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 104 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose melts were separately subjected to phenol/chloroform extraction and ethanol precipitation, to purify the DNA fragments. Finally, these were separately dissolved in 10  $\mu$ l of TE.

The DNA fragment of about 770 bp obtained from the

clone No. 107 and the DNA fragment of about 3.8 kbp obtained from the clone No. 104 were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced  
5 into competent cells of *E.coli* HB101 (manufactured by Toyobo Co., Ltd.). Thus was obtained a transformant No. 117.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

10 Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are  
15 shown in Table 117, in which it is known that the 19<sup>th</sup> Ala in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Val, the 71<sup>st</sup> Arg in the same was substituted with His, the 126<sup>th</sup> Phe in the same was substituted with Tyr, while the 37<sup>th</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was  
20 substituted with Leu, the 108<sup>th</sup> Glu in the same was substituted with Asp, and the 200<sup>th</sup> Ala in the same was substituted with Glu.

[Table 117]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 117	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	71 <sup>st</sup> position in $\alpha$ -subunit	Arg	His	CGT	CAT
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Leu	TTC	CTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

[Example 78]

Construction (118) of the Substituted Amino Acid Having Nitrile Hydratase Activity

- 5 This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 104 and that from the clone No. 108 still had the nitrile hydratase activity.



10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final concentration of 100 µg/ml. On the medium, one platinum  
5 loop of the cells of the clone No. 104 as prepared in Example 64 and the clone No. 108 as prepared in Example 68 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting culture was put into a suitable centrifugal tube, and this  
10 was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. From the cells extracted the plasmid DNA of the clone No. 104 and the plasmid DNA of the clone No. 108 through alkaline SDS extraction.

15 The plasmid DNA from the clone No. 108 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose  
20 concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 104 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel  
25 electrophoresis (using Type VII low-melting-point agarose,

manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose melts were separately subjected to phenol/chloroform extraction and ethanol precipitation, to purify the DNA fragments. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 108 and the DNA fragment of about 3.8 kbp obtained from the clone No. 104 were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced into competent cells of *E.coli* HB101 (manufactured by Toyobo Co.,Ltd.). Thus was obtained a transformant No. 118.

Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an

Autosequencer 373A manufactured by ABI. The results are shown in Table 118, in which it is known that the 19<sup>th</sup> Ala in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted with Val, the 71<sup>st</sup> Arg in the same was substituted with His, the 126<sup>th</sup> Phe in the same was substituted with Tyr, while the 37<sup>th</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Val, the 108<sup>th</sup> Glu in the same was substituted with Asp, and the 200<sup>th</sup> Ala in the same was substituted with Glu.

10 [Table 118]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 118	19 <sup>th</sup> position in $\alpha$ -subunit	Ala	Val	GCG	GTG
	71 <sup>st</sup> position in $\alpha$ -subunit	Arg	His	CGT	CAT
	126 <sup>th</sup> position in $\alpha$ -subunit	Phe	Tyr	TTC	TAC
	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT

	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC
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[Example 79]

Construction (119) of the Substituted Amino Acid Having  
Nitrile Hydratase Activity

5           This is to demonstrate that the mutant with the  
substituted amino acid sequence comprising both of the  
mutated position from the clone No. 105 and that from the  
clone No. 109 still had the nitrile hydratase activity.

10           10 ml of a liquid LB medium was put into a 30 ml test  
tube, and sterilized by autoclaving at 121°C for 20 minutes.  
Ampicillin was added to this medium to have a final  
concentration of 100  $\mu$ g/ml. On the medium, one platinum  
loop of the cells of the clone No. 105 as prepared in  
Example 65 and the clone No. 109 as prepared in Example 69  
15           was inoculated and incubated therein at 37°C for about 20  
hours with stirring at 300 rpm. 1 ml of the resulting  
culture was put into a suitable centrifugal tube, and this  
was subjected to centrifugation (15,000 rpm, 5 minutes) to  
separate the cells from the culture. From the cells  
20           extracted the plasmid DNA of the clone No. 105 and the  
plasmid DNA of the clone No. 109 through alkaline SDS  
extraction.

The plasmid DNA from the clone No. 109 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 105 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose melts were separately subjected to phenol/chloroform extraction and ethanol precipitation, to purify the DNA fragments. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 109 and the DNA fragment of about 3.8 kbp obtained from the clone No. 105 were subjected to DNA ligation, using

a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced into competent cells of *E.coli* HB101 (manufactured by Toyobo Co.,Ltd.). Thus was obtained a transformant No. 119.

5        Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

         Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
10    nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 119, in which it is known that the 36<sup>th</sup> Thr in the  $\alpha$ -subunit in the wild nitrile hydratase was substituted  
15    with Met, the 148<sup>th</sup> Gly in the same was substituted with Asp, the 204<sup>th</sup> Val in the same was substituted with Arg, while the 41<sup>st</sup> Phe in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Ile, the 51<sup>st</sup> Phe in the same was substituted with Val, and the 108<sup>th</sup> Glu in the same was  
20    substituted with Asp.

[Table 119]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 119	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	ACG	ATG
	148 <sup>th</sup> position in $\alpha$ -subunit	Gly	Asp	GGC	GAC
	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Arg	GTC	CGC
	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Ile	TTC	ATC
	51 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	TTC	GTC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT

[Example 80]

Construction (120) of the Substituted Amino Acid Having

5 Nitrile Hydratase Activity

This is to demonstrate that the mutant with the substituted amino acid sequence comprising both of the mutated position from the clone No. 98 and that from the

clone No. 100 still had the nitrile hydratase activity.

10 ml of a liquid LB medium was put into a 30 ml test tube, and sterilized by autoclaving at 121°C for 20 minutes. Ampicillin was added to this medium to have a final

5 concentration of 100 µg/ml. On the medium, one platinum loop of the cells of the clone No. 98 as prepared in Example 59 and the clone No. 100 as prepared in Reference Example 40 was inoculated and incubated therein at 37°C for about 20 hours with stirring at 300 rpm. 1 ml of the resulting  
10 culture was put into a suitable centrifugal tube, and this was subjected to centrifugation (15,000 rpm, 5 minutes) to separate the cells from the culture. From the cells extracted the plasmid DNA of the clone No. 98 and the plasmid DNA of the clone No. 100 through alkaline SDS  
15 extraction.

The plasmid DNA from the clone No. 100 was cleaved by means of restriction endonucleases EcoRI and NotI at the cleaving sites thereof, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose,  
20 manufactured by Sigma Corporation; at an agarose concentration of 1.0%), through which the DNA fragment of about 770 bp was cut out of the agarose gel. In the same manner, the plasmid DNA from the clone No. 98 was cleaved by means of restriction endonucleases EcoRI and NotI at the  
25 cleaving sites thereof, and then subjected to agarose gel



electrophoresis (using Type VII low-melting-point agarose, manufactured by Sigma Corporation; at an agarose concentration of 0.7 %), through which the DNA fragment of about 3.8 kbp was cut out of the agarose gel. Both the  
5 thus-cut agarose fragments (about 0.1 g each) were finely pulverized, then separately suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragments were completely melted. The resulting agarose melts were separately subjected to phenol/chloroform  
10 extraction and ethanol precipitation, to purify the DNA fragments. Finally, these were separately dissolved in 10 µl of TE.

The DNA fragment of about 770 bp obtained from the clone No. 100 and the DNA fragment of about 3.8 kbp obtained  
15 from the clone No. 98 were subjected to DNA ligation, using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.), to construct a plasmid. This plasmid DNA was introduced into competent cells of *E.coli* HB101 (manufactured by Toyobo Co., Ltd.). Thus was obtained a transformant No. 120.

20        Thereafter, the addition rate and the selectivity were determined in the same manner as in Reference Example 1, and the conversion and the selectivity were 100%, respectively.

Moreover, the plasmid was prepared from the cells by alkaline SDS extraction. Next, the base sequence of the  
25 nitrile hydratase gene was determined by the dideoxy chain

termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. The results are shown in Table 120, in which it is known that the 148<sup>th</sup> Gly in the  $\alpha$ -subunit in the wild nitrile hydratase was

5 substituted with Asp, the 204<sup>th</sup> Val in the same was substituted with Arg, while the 108<sup>th</sup> Glu in the  $\beta$ -subunit in the wild nitrile hydratase was substituted with Asp, and the 200<sup>th</sup> Ala in the same was substituted with Glu.

[Table 120]

Clone Number	Mutated Site	Change in Amino Acid Sequence		Change in Base Sequence	
		Wild Type	Mutant	Wild Type	Mutant
No. 120	148 <sup>th</sup> position in $\alpha$ -subunit	Gly	Asp	GGC	GAC
	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Arg	GTC	CGC
	108 <sup>th</sup> position in $\beta$ -subunit	Glu	Asp	GAG	GAT
	200 <sup>th</sup> position in $\beta$ -subunit	Ala	Glu	GCC	GAC

10

[Example 81]

Preparation of genomic DNA from *Rhodococcus rhodochrous*

strain J1

In a 500 ml Erlenmeyer flask with baffles, 100 ml of a medium of the following composition was prepared and sterilized by autoclaving at 121°C for 20 minutes.

5 Medium composition;

Glucose: 10.0 g/L, potassium dihydrogen phosphate: 0.5 g/L, dipotassium hydrogen phosphate: 0.5 g/L, magnesium sulfate·heptahydrate: 0.5 g/L, yeast extract: 1.0 g/L, peptone: 7.5 g/L, urea: 7.5 g/L, cobalt  
10 chloride·hexahydrate: 10.0 mg/L, pH 7.2

On this medium, one platinum loop of the cells of *Rhodococcus rhodochrous* strain J1 (deposited with the above-mentioned depository authority under the deposit number of FERM BP-1478 under the Budapest Treaty for the international  
15 recognition of the deposit of microorganisms for the purposes of patent procedure and may be distributed to anyone on request) as described in Patent Document 1 was inoculated and incubated therein at 30°C for 72 hours with stirring at 130 rpm. The cells were separated from the  
20 resulting culture by centrifugation (15,000 G × 15 minutes). The cells were resuspended in 50 ml of physiological saline, and then subjected to another centrifugation, thereby wet cells being obtained.

To 2 g of the wet cells obtained above, 40 ml of an  
25 aqueous solution (pH 8.0) of 50 mM EDTA·2Na containing 0.15

M NaCl was added to prepare a cell suspension, which was boiled at 90°C for 10 minutes. The resulting suspension was cooled to 37°C, to which was added 100 mg of egg white lysozyme, and was kept at 37°C for 1 hour. To this, next, 5 30 mg of zymolyase of 20,000 U/mg was added, followed by keeping at 37°C for 1 hour. Subsequently, 5 mg of proteinase K of 20 U/mg was added to this, followed by keeping at 37°C for 1 hour. Further, 2 ml of 10% SDS solution was added to this, followed by keeping at 65°C for 10 1 hour, and then the mixture was immediately subjected to phenol/chloroform extraction. First, 42 ml of a phenol solution saturated with TE (10 mM Tris-HCl aqueous solution containing 1 mM EDTA·2Na; pH 8.0) was added to the reaction mixture, which was gently stirred. This was subjected to 15 centrifugation (3,000 rpm, 10 minutes) to separate it into an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, 21 ml of the aforementioned TE-saturated phenol solution and 21 ml of chloroform were added, and the mixture was gently stirred 20 again. Then, this was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was further collected. To this aqueous phase, 42 ml of chloroform was added, and the mixture was gently stirred. Then, this was 25 subjected to centrifugation (3,000 rpm, 10 minutes) again to

separate an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, 4 ml of a TE solution containing 1.1 M NaCl and 92 ml of ethanol were added, and the mixture was left as such at room temperature for a while. Then, thus precipitated yarn-like DNA was collected by winding it around a glass rod. This was dehydrated through treatment with aqueous solutions of 70%, 80% and 90% ethanol in that order and then dried in the air. Then, thus collected DNA was redissolved in 40 ml of a TE solution. To this, 30 µg of RNase A was added, followed by keeping at 37°C for 1 hour. Then, this was partially cleaved by means of a restriction endonuclease BamHI. The DNA thus partially cleaved was again purified by phenol extraction/chloroform extraction and ethanol precipitation, and it was dissolved in a TE solution to a final concentration of 1.0 µg/ml.

[Example 82]

Preparation of nitrile hydratase gene from the genomic DNA of *Rhodococcus rhodochrous* strain J1 using PCR

Based on the base sequence of the nitrile hydratase gene as disclosed Patent Document 2 and Non-patent Document 1, the primers having the sequence as set forth in SEQ ID NOs: 105 and 106 in the Sequence Listing were synthesized. PCR was performed using 3 µg of the partially cleaved

chromosome DNA as prepared in Example 81 as the template.

In the PCR reaction, a system of 50  $\mu$ l containing 200 ng of each primer and 1 U of KOD polymerase (manufactured by

Toyobo Co., Ltd.) was subjected to 40 PCR cycles comprising

5 thermal denaturation (98°C) for 15 seconds, annealing (58°C) for 15 seconds and chain extension (68°C) for 2 minutes.

After completion of the PCR reaction, the reaction mixture

was subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, product by Sigma Corporation;

10 agarose concentration 0.8% by weight) and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product of about 1.3 Kbp.

Subsequently, an agarose fragment comprising only a DNA

15 fragment of about 1.3 kbp was cut out of the agarose gel.

The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to

20 phenol/chloroform extraction and ethanol precipitation.

First, to this, 1 ml of a phenol solution saturated with TE (an aqueous solution of 10 mM Tris-HCl containing 1 mM EDTA·2Na; pH 8.0) was added, and the mixture was gently stirred. This was subjected to centrifugation (3,000 rpm,

25 10 minutes) to separate an aqueous phase and an organic

phase, and only the aqueous phase was collected. After repeating this operation three times, to the obtained aqueous phase, 0.4 ml of the TE-saturated phenol solution and 0.4 ml of chloroform were added, and the mixture was again gently stirred. Then, this was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was further collected. To this aqueous phase, 0.8 ml of chloroform was added, and the mixture was gently stirred. This was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, 80  $\mu$ l of a TE solution containing 1.1 M NaCl and 1.7 ml of ethanol were added, and after being stood still at -80°C for 30 minutes, the mixture was subjected to centrifugation (15,000 rpm, 20 minutes, 4°C) to recover DNA fragments. These DNA fragments were dried in the air and finally dissolved in 10  $\mu$ l of TE.

After the purified amplified DNA fragment of about 1.3 kbp was cleaved by means of restriction endonucleases EcoRI and ScaI, an assay of the DNA amplification product was carried out by agarose gel electrophoresis (using Type VII low-melting-point agarose, product by Sigma Corporation; agarose concentration 0.8% by weight), and it was possible to confirm the presence of a DNA of about 1.3 Kbp. An

agarose fragment comprising only the DNA fragment of about 1.3 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction and ethanol precipitation. First, to this, 1 ml of a phenol solution saturated with TE (an aqueous solution of 10 mM Tris-HCl containing 1 mM EDTA·2Na; pH 8.0) was added, and the mixture was gently stirred. This was subjected to centrifugation (3,000 rpm, 10 minutes) to separate an aqueous phase and an organic phase, and only the aqueous phase was collected. After repeating this operation three times, to the obtained aqueous phase, 0.4 ml of the TE-saturated phenol solution and 0.4 ml of chloroform were added, and the mixture was again gently stirred. Then, this was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, 0.8 ml of chloroform was added, and the mixture was gently stirred. This was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, 80 µl of a TE solution containing 1.1 M NaCl and 1.7 ml of



ethanol were added, and after being stood still at -80°C for 30 minutes, the mixture was subjected to centrifugation (15,000 rpm, 20 minutes, 4°C) to recover the DNA fragment. This DNA fragment was dried in the air and finally dissolved in 10 µl of TE.

[Example 83]

Preparation of plasmid vector to express the *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase gene

In a 500 ml Erlenmeyer flask with baffles, 100 ml of a liquid LB medium of the following composition containing 40 µg/ml of ferric sulfate·heptahydrate and 10 µg/ml of cobalt chloride·hexahydrate was prepared and sterilized by autoclaving at 121°C for 20 minutes.

Medium composition;

Yeast extract: 5.0 g/L, polypeptone: 10.0 g/L, NaCl: 5.0 g/L, cobalt chloride·hexahydrate: 10.0 mg/L, ferric sulfate·heptahydrate: 40.0 mg/L, pH 7.5

To this medium, ampicillin was added to a final concentration of 100 µg/ml. Subsequently, one platinum loop of the cells of MT10822 (FERM BP-5785) as described in Patent Document 3 was inoculated and incubated therein at 37°C for 16 hours with stirring at 130 rpm. The cells were separated from the resulting culture by centrifugation (15,000 G × 15 minutes). Subsequently, the cells were

resuspended in 50 ml of physiological saline, and then subjected to another centrifugation, thereby wet cells being obtained. The plasmid DNA pPT-DB1 (Figure 1) was prepared from these wet cells by alkaline SDS extraction, and the  
5 product was subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixture was subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNA, and the DNA was dissolved in a TE solution to a final concentration of 1.0  
10 µg/µl.

After 1 µg of the purified plasmid was cleaved by means of restriction endonucleases EcoRI and Eco47III, an assay of the DNA amplification product was carried out by agarose gel electrophoresis (using Type VII low-melting-point agarose,  
15 product by Sigma Corporation; agarose concentration 0.8% by weight), and it was possible to confirm the presence of DNAs of about 3.3 kbp and of about 1.3 kbp. An agarose fragment comprising only the DNA fragment of about 3.3 kbp was cut out of the agarose gel. The thus-cut agarose fragment  
20 (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction and ethanol precipitation. First, to this, 1 ml of a phenol  
25 solution saturated with TE (an aqueous solution of 10 mM

Tris-HCl containing 1 mM EDTA·2Na; pH 8.0) was added, and the mixture was gently stirred. This was subjected to centrifugation (3,000 rpm, 10 minutes) to separate an aqueous phase and an organic phase, and only the aqueous phase was collected. After repeating this operation three times, to the obtained aqueous phase, 0.4 ml of the TE-saturated phenol solution and 0.4 ml of chloroform were added, and the mixture was again gently stirred. Then, this was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was further collected. To this aqueous phase, 0.8 ml of chloroform was added, and the mixture was gently stirred. This was subjected to centrifugation (3,000 rpm, 10 minutes) again to separate an aqueous phase and an organic phase, and only the aqueous phase was collected. To this aqueous phase, 80 µl of a TE solution containing 1.1 M NaCl and 1.7 ml of ethanol were added, and after being stood still at -80°C for 30 minutes, the mixture was subjected to centrifugation (15,000 rpm, 20 minutes, 4°C) to recover the DNA fragment. This DNA fragment was dried in the air and finally dissolved in 10 µl of TE.

[Example 84]

Construction of transformant to activate and express the

Rhodococcus rhodochrous strain J1-derived nitrile hydratase

A mixture of the DNA fragment of about 1.3 kbp which was prepared in Example 82 by cleavage by means of EcoRI and ScaI, and the DNA fragment of about 3.3 kbp which was prepared in Example 83 by cleavage by means of EcoRI and Eco47III was subjected to DNA ligation. A competent cell of E. coli HB101 (manufactured by Toyobo Co., Ltd.) was transformed with the reaction product to obtain transformant No. 200.

In a 500 ml Erlenmeyer flask with baffles, 100 ml of a liquid LB medium containing 40 µg/ml of ferric sulfate·heptahydrate and 10 µg/ml of cobalt chloride·hexahydrate was prepared and sterilized by autoclaving at 121°C for 20 minutes. To this medium, ampicillin was added to a final concentration of 100 µg/ml. Subsequently, one platinum loop of the transformant No. 200 was inoculated and incubated therein at 37°C for 20 hours with stirring at 130 rpm. The transformants were separated from the resulting culture by centrifugation (5,000 G × 15 minutes). The transformants were resuspended in 50 ml of physiological saline and then subjected to another centrifugation (5,000 G × 15 minutes), thereby the transformants being isolated.

0.1 g of thus isolated transformants were suspended in 20 ml of an aqueous solution (pH 7.0) of 50 mM potassium

phosphate. To this, 0.5 ml of acrylonitrile or methacrylonitrile was added, and this mixture was gently stirred at 30°C for 1 hour to react. After completion of the reaction, an analysis of the reaction solution was carried out with HPLC, and it was found that the reaction solution contained only an amide compound (acrylamide or methacrylamide) of a molar amount corresponding to the amount of the added nitrile compound (acrylonitrile or methacrylonitrile), and that the nitrile compound (acrylonitrile or methacrylonitrile) and the corresponding organic acid (acrylic acid or methacrylic acid) were absent. Thereafter, the conversion and the selectivity were 100%, respectively.

Further, a plasmid was prepared from this isolated transformant by alkaline SDS extraction, and the product was subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixture was subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNA, and the DNA was dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequence of the nitrile hydratase gene was determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI. As a result, it was confirmed that the plasmid contained in its sequence the ORF which codes for the nitrile hydratase

activating protein as set forth in SEQ ID NO: 103 in the Sequence Listing and the ORF which codes for the *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase as set forth in SEQ ID NO: 104 in the Sequence Listing. This plasmid was  
5 named as pJ1H-DB1 (Figure 2).

[Example 85]

Extraction of the target of mutation from nitrile hydratase before modification (1)

10       As the subject of the method for modification, *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase was used as an example. Extraction of the target was carried out employing the method of specifying the amino acid residues which serve as the target of mutation as  
15 described in Claims 56 to 62 as an example. In the alignment of amino acid sequences for the method for specification as described in Claims 56 and 57, DNASIS manufactured by Hitach Software Engineering co., Ltd. was used. In the modeling of stereostructure based on the  
20 alignment of amino acid sequences for the method for specification as described in Claims 58 to 62, Modeler or Homology produced by Accelrys, Inc. was used.

As a result, in all cases, the extracted amino acid residues contained the 48<sup>th</sup> Trp of the amino acid sequence of  
25 the  $\beta$ -subunit, which is one of the two polypeptides

constituting the nitrile hydratase. Thus, it was decided to use the 48<sup>th</sup> Trp of the amino acid sequence of the  $\beta$ -subunit of the *Rhodococcus rhodochrous* stain J1-derived nitrile hydratase as the example for the target of mutation.

5

[Example 86]

Extraction of the target of mutation from nitrile hydratase before modification (2)

As the subject of the method for modification,  
10 *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase was used as an example. Extraction of the target was carried out employing the method of specifying the amino acid residues which serve as the target of mutation as described in Claims 56 to 62 as an example. In the  
15 alignment of amino acid sequences for the method for specification as described in Claims 56 and 57, DNASIS manufactured by Hitach Software Engineering co., Ltd. was used. In the modeling of stereostructure based on the alignment of amino acid sequences for the method for  
20 specification as described in Claims 58 to 62, Modeler or Homology produced by Accelrys, Inc. was used.

As a result, it was decided to use, among the amino acid residues extracted as the amino acid residues that are present in the region involved with forming a channel  
25 through which a substrate passes from the outside of the

enzyme toward the active center or a product passes from the active center toward the outside of the enzyme, the 36<sup>th</sup> Thr and 48<sup>th</sup> Asn of the amino acid sequence of the  $\alpha$ -subunit which is one of the two polypeptides constituting the nitrile hydratase, and the 32<sup>nd</sup> Val, 33<sup>rd</sup> Ala, 37<sup>th</sup> Phe, 40<sup>th</sup> Thr, 41<sup>st</sup> Phe, 46<sup>th</sup> Met, 48<sup>th</sup> Leu, 51<sup>st</sup> Phe, 61<sup>st</sup> Ala, 72<sup>nd</sup> Trp, 112<sup>th</sup> Lys, 118<sup>th</sup> Phe and 127<sup>th</sup> Leu of the amino acid sequence of the  $\beta$ -subunit which is another polypeptide, as the representative examples for the target of mutation, respectively.

[Example 87]

Extraction of the target of mutation from nitrile hydratase before modification (3)

As the subject of the method for modification, *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase was used as an example. Extraction of the target was carried out employing the method of specifying the amino acid residues which serve as the target of mutation as described in Claims 56 to 62 as an example. In the alignment of amino acid sequences for the method for specification as described in Claims 56 and 57, DNASIS manufactured by Hitach Software Engineering co., Ltd. was used. In the modeling of stereostructure based on the alignment of amino acid sequences for the method for



specification as described in Claims 58 to 62, Modeler or Homology produced by Accelrys, Inc. was used.

As a result, it was decided to use, among the amino acid residues extracted as the amino acid residues that are present in the region forming an associative interface between the  $\alpha$ -subunit and the  $\beta$ -subunit which is involved with the formation of dimers or in the region forming an interface which is involved with the association of dimers, the 36<sup>th</sup> Thr, 148<sup>th</sup> Gly, 188<sup>th</sup> Thr and 204<sup>th</sup> Val of the amino acid sequence of the  $\alpha$ -subunit which is one of the two polypeptides constituting the nitrile hydratase, and the 10<sup>th</sup> Thr, 32<sup>nd</sup> Val, 33<sup>rd</sup> Ala, 112<sup>th</sup> Lys, 118<sup>th</sup> Phe, 127<sup>th</sup> Leu, 146<sup>th</sup> Arg, 150<sup>th</sup> Ala, 160<sup>th</sup> Arg, 168<sup>th</sup> Thr, 171<sup>st</sup> Lys, 176<sup>th</sup> Tyr, 186<sup>th</sup> Leu, 217<sup>th</sup> Asp and 218<sup>th</sup> Cys of the amino acid sequence of the  $\beta$ -subunit which is another polypeptide, as the representative examples for the target of mutation, respectively.

[Example 88]

Introduction of mutation for construction of modified enzyme (1)

In order to introduce mutation to the amino acid sequence of the polypeptides constituting nitrile hydratase, introduction of site-specific mutation was performed using a "LA PCR in vitro mutagenesis Kit" (manufactured by Takara

Shuzo Co., Ltd.). The "LA PCR in vitro mutagenesis Kit" is simply referred to as the kit. In the following examples, the kit was handled on the basis of the principle thereof and in accordance with the manufacturer's instructions for the kit.

Onto a plasmid: pJ1H-DB1 containing the ORF that codes for the *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase as constructed in Example 84, mutation was performed to change the 48<sup>th</sup> Trp of the amino acid sequence of the  $\beta$ -subunit, which is the target of mutation as extracted in Example 85, to another amino acid.

Using 10 ng of pJ1H-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 110 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO:

108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
(having the sequence as set forth in SEQ ID NO: 109 in the  
Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
5 used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l  
of the reaction mixture was subjected to agarose gel  
electrophoresis (where the agarose concentration was 1.0% by  
10 weight), and an assay of the DNA amplification product was  
carried out. This assay revealed the production of the  
amplified DNA products in the both PCR reactions. From each  
of these PCR reaction mixtures, the excess primers and dNTP  
were removed using Microcon 100 (manufactured by Takara  
15 Shuzo Co., Ltd.), and then TE was added to each of the  
mixtures to prepare 50  $\mu$ l each of TE solutions. An  
annealing solution of 47.5  $\mu$ l in total containing 0.5  $\mu$ l of  
both of the above TE solutions (for the composition of the  
system, the manufacturer's instructions for the kit were  
20 followed) was prepared, and this solution was subjected to  
annealing by performing thermal denaturation of the solution  
at 98°C for 10 minutes, subsequently cooling the solution to  
37°C at a constant cooling rate over a period of 60 minutes,  
and then maintaining it at 37°C for 15 minutes.

25 To thus annealed solution, 0.5  $\mu$ l of TAKARA LA Taq was

added, and the solution was heated at 72°C for 3 minutes, thus completing the formation of heterologous double-stranded DNA. This was then subjected to PCR reaction No. 3. For PCR reaction No. 3, a reaction system of 50 µl in total  
5 comprising 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) was used, and the reaction consisted of 25 PCR cycles, in which one  
10 PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes.

After completion of the PCR reaction No. 3, 5 µl of the reaction mixture was subjected to agarose gel  
15 electrophoresis (using Type VII low-melting-point agarose, product by Sigma Corporation; agarose concentration 0.8% by weight), and an assay of the DNA amplification product was carried out, thereby it being possible to confirm the presence of an amplified DNA product of about 1.9 Kbp. An  
20 agarose fragment comprising only the DNA fragment of about 1.9 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted.  
25 The resulting agarose melt was subjected to

phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10  $\mu$ l of TE.

After the purified amplified DNA fragment of about 1.9 kbp was cleaved by means of restriction endonucleases EcoRI and HindIII, this mixture treated with restriction endonucleases was subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10  $\mu$ l of TE.

10 Likewise, pJ1H-DB1 was cleaved by means of EcoRI and HindIII, and the mixture was subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, product by Sigma Corporation; agarose concentration of 0.7%). An agarose fragment comprising only the DNA fragment of about 2.7 kbp

15 was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction

20 and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10  $\mu$ l of TE.

Thus obtained DNA fragments of about 1.9 kbp and of about 2.7 kbp were subjected to DNA ligation. A competent cell of *E. coli* HB101 (manufactured by Toyobo Co., Ltd.) was

25 transformed with the reaction product to obtain a number of

transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour.

Subsequently, the mixtures were subjected to phenol

5 extraction/chloroform extraction, and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an  
10 Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 48<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of

15 *Rhodococcus rhodochrous* strain J1-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Trp, as compared with pJ1H-DB1.

In Table 121 below, the numbers of the obtained  
20 transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 121]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 201	48 <sup>th</sup> position in β-subunit	Trp	Tyr	tgg	tat
No. 202	48 <sup>th</sup> position in β-subunit	Trp	Val	tgg	gtg
No. 203	48 <sup>th</sup> position in β-subunit	Trp	Ala	tgg	gcg
No. 204	48 <sup>th</sup> position in β-subunit	Trp	Gly	tgg	ggg

[Example 89]

Introduction of mutation for construction of modified enzyme  
(2)

- 5        Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 36<sup>th</sup> Thr of the amino

acid sequence of the  $\alpha$ -subunit, which is the target of mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
5 different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 111 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence  
10 Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension  
15 (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the  
20 Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

After completion of the PCR reaction Nos. 1 and 2, 5  $\mu$ l  
25 of the reaction mixture was subjected to agarose gel



electrophoresis (where the agarose concentration was 1.0% by weight), and an assay of the DNA amplification product was carried out. This assay revealed the production of the amplified DNA products in the both PCR reactions. From each  
5 of these PCR reaction mixtures, the excess primers and dNTP were removed using Microcon 100 (manufactured by Takara Shuzo Co., Ltd.), and then TE was added to each of the mixtures to prepare 50  $\mu$ l each of TE solutions. An annealing solution of 47.5  $\mu$ l in total containing 0.5  $\mu$ l of  
10 both of the above TE solutions (for the composition of the system, the manufacturer's instructions for the kit were followed) was prepared, and this solution was subjected to annealing by performing thermal denaturation of the solution at 98°C for 10 minutes, subsequently cooling the solution to  
15 37°C at a constant cooling rate over a period of 60 minutes, and then maintaining it at 37°C for 15 minutes.

To thus annealed solution, 0.5  $\mu$ l of TAKARA LA Taq was added, and the solution was heated at 72°C for 3 minutes, thus completing the formation of heterologous double-  
20 stranded DNA. This was then subjected to PCR reaction No. 3. For PCR reaction No. 3, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set  
25 forth in SEQ ID NO: 109 in the Sequence Listing) was used,

and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes.

5        After completion of the PCR reaction No. 3, 5 µl of the reaction mixture was subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, product by Sigma Corporation; agarose concentration 0.8% by weight), and an assay of the DNA amplification product was  
10       carried out, thereby it being possible to confirm the presence of an amplified DNA product of about 1.9 Kbp. An agarose fragment comprising only the DNA fragment of about 1.9 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized,  
15       suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was  
20       finally dissolved in 10 µl of TE.

      After the purified amplified DNA fragment of about 1.9 kbp was cleaved by means of restriction endonucleases EcoRI and HindIII, this mixture treated with restriction endonucleases was subjected to phenol/chloroform extraction  
25       and ethanol precipitation to purify the DNA fragment. Thus

purified DNA fragment was finally dissolved in 10  $\mu$ l of TE. Likewise, pPT-DB1 was cleaved by means of EcoRI and HindIII, and then subjected to agarose gel electrophoresis (using Type VII low-melting-point agarose, product by Sigma Corporation; agarose concentration of 0.7%). An agarose fragment comprising only the DNA fragment of about 2.7 kbp was cut out of the agarose gel. The thus-cut agarose fragment (about 0.1 g) was finely pulverized, suspended in 1 ml of a TE solution, and kept at 55°C for 1 hour, whereby the agarose fragment was completely melted. The resulting agarose melt was subjected to phenol/chloroform extraction and ethanol precipitation to purify the DNA fragment. Thus purified DNA fragment was finally dissolved in 10  $\mu$ l of TE.

Thus obtained DNA fragments of about 1.9 kbp and of about 2.7 kbp were subjected to DNA ligation. A competent cell of *E. coli* HB101 (manufactured by Toyobo Co., Ltd.) was transformed with the reaction product to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0  $\mu$ g/ $\mu$ l. The base sequences thereof were determined by the dideoxy chain

termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 36<sup>th</sup> amino acid in the amino acid sequence of the  $\alpha$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Thr, as compared with pPT-DB1.

In Table 122 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

15 [Table 122]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 40	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Met	acg	atg
No. 40a	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Ser	acg	tcg
No. 40b	36 <sup>th</sup> position	Thr	Ala	acg	gcg

	in $\alpha$ -subunit				
No. 40c	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Gly	acg	ggg
No. 40d	36 <sup>th</sup> position in $\alpha$ -subunit	Thr	Trp	acg	tgg

[Example 90]

Introduction of mutation for construction of modified enzyme  
(3)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 48<sup>th</sup> Asn of the amino  
acid sequence of the  $\alpha$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

          Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 112 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was

used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a

5 reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the  
10 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids  
15 were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were  
20 each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the  
25 above-obtained transformants were such that the base

sequence corresponding to the codon encoding the 48<sup>th</sup> amino acid in the amino acid sequence of the  $\alpha$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Asn, as compared with pPT-DB1.

In Table 123 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 123]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 40e	48 <sup>th</sup> position in $\alpha$ -subunit	Asn	Gln	aac	caa
No. 40f	48 <sup>th</sup> position in $\alpha$ -subunit	Asn	Glu	aac	gaa

[Example 91]

Introduction of mutation for construction of modified enzyme

15 (4)

Onto a plasmid: pPT-DB1 containing the ORF that codes

for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 71<sup>st</sup> Arg of the amino acid sequence of the  $\alpha$ -subunit, which is the target of  
5 mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
10 the primer having the sequence as forth in SEQ ID NO: 113 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
15 used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an  
20 MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed). was  
25 used, and the reaction was carried out following the same



procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the  
5 plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of  
10 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base  
15 sequence corresponding to the codon encoding the 71<sup>st</sup> amino acid in the amino acid sequence of the α-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Arg, as compared  
20 with pPT-DB1.

In Table 124 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

25 [Table 124]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 41	71 <sup>st</sup> position in $\alpha$ -subunit	Arg	His	cgt	cat

[Example 92]

Introduction of mutation for construction of modified enzyme (5)

5       Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 148<sup>th</sup> Gly of the amino acid sequence of the  $\alpha$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 114 in the Sequence Listing and 50 pmol of an M13 primer M4 (having

the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which  
5 one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO:  
10 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and  
20 incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the  
25 dideoxy chain termination method using a sequencing kit and

an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 148<sup>th</sup> amino acid in the amino acid sequence of the  $\alpha$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Gly, as compared with pPT-DB1.

In Table 125 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 125]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 42	148 <sup>th</sup> position in $\alpha$ -subunit	Gly	Asp	ggc	gac

[Example 93]

Introduction of mutation for construction of modified enzyme  
(6)

Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
5 hydratase as constructed from MT10822 in Example 83;  
mutation was performed to change the 188<sup>th</sup> Thr of the amino  
acid sequence of the  $\alpha$ -subunit, which is the target of  
mutation as extracted in Example 86 or 87, to another amino  
acid.

10 Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
the primer having the sequence as forth in SEQ ID NO: 115 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
15 the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
20 seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 2 minutes. For the PCR reaction No. 2, a  
reaction system of 50  $\mu$ l in total comprising 50 pmol of an  
MUT4 primer (having the sequence as set forth in SEQ ID NO:  
108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
25 (having the sequence as set forth in SEQ ID NO: 109 in the

Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

5        Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures  
10       were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and  
15       an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 188<sup>th</sup> amino acid in the amino acid sequence of the α-subunit of  
20       *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Thr, as compared with pPT-DB1.

In Table 126 below, the numbers of the obtained  
25       transformants as well as the corresponding mutated sites,

changes in amino acid sequence and changes in base sequence are given.

[Table 126]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 42a	188 <sup>th</sup> position in $\alpha$ -subunit	Thr	Gly	acc	ggc

5 [Example 94]

Introduction of mutation for construction of modified enzyme (7)

Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
10 hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 204<sup>th</sup> Val of the amino acid sequence of the  $\alpha$ -subunit, which is the target of mutation as extracted in Example 86 or 87, to another amino acid.

15 Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No.

1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 116 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were



each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

5 As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 204<sup>th</sup> amino acid in the amino acid sequence of the  $\alpha$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase  
10 had been changed to a base sequence corresponding to the codon encoding an amino acid other than Val, as compared with pPT-DB1.

In Table 127 below, the numbers of the obtained transformants as well as the corresponding mutated sites,  
15 changes in amino acid sequence and changes in base sequence are given.

[Table 127]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 43	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Arg	gtc	cgc

No. 44	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Lys	gtc	aaa
No. 45	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Trp	gtc	tgg
No. 46	204 <sup>th</sup> position in $\alpha$ -subunit	Val	Thr	gtc	acc

[Example 95]

Introduction of mutation for construction of modified enzyme  
(8)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 10<sup>th</sup> Thr of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

          Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 117 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the

manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the

above-obtained transformants were such that the base sequence corresponding to the codon encoding the 10<sup>th</sup> amino acid in the amino acid sequence of the  $\beta$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase

5 had been changed to a base sequence corresponding to the codon encoding an amino acid other than Thr, as compared with pPT-DB1.

In Table 128 below, the numbers of the obtained transformants as well as the corresponding mutated sites,  
10 changes in amino acid sequence and changes in base sequence are given.

[Table 128]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 47	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Asp	acc	gac
No. 48	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Glu	acc	gaa
No. 49	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Trp	acc	tgg
No. 50	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Gly	acc	ggc

No. 51	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Tyr	acc	tac
No. 52	10 <sup>th</sup> position in $\beta$ -subunit	Thr	Cys	acc	tgc

[Example 96]

Introduction of mutation for construction of modified enzyme  
(9)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 32<sup>nd</sup> Val of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

          Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 118 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
20 used, and the reaction consisted of 25 PCR cycles, in which

one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an  
5 MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the  
15 plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of  
20 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base  
25 sequence corresponding to the codon encoding the 32<sup>nd</sup> amino

acid in the amino acid sequence of the  $\beta$ -subunit of  
*Pseudonocardia thermophila* JCM3095-derived nitrile hydratase  
had been changed to a base sequence corresponding to the  
codon encoding an amino acid other than Val, as compared  
5 with pPT-DB1.

In Table 129 below, the numbers of the obtained  
transformants as well as the corresponding mutated sites,  
changes in amino acid sequence and changes in base sequence  
are given.

10 [Table 129]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 53	32 <sup>nd</sup> positon in $\beta$ - subunit	Val	Gly	gtc	ggc

[Example 97]

Introduction of mutation for construction of modified enzyme  
(10)

15       Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile

hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 33<sup>rd</sup> Ala of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
mutation as extracted in Example 86 or 87, to another amino  
5 acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
the primer having the sequence as forth in SEQ ID NO: 119 in  
10 the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
15 one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 2 minutes. For the PCR reaction No. 2, a  
reaction system of 50  $\mu$ l in total comprising 50 pmol of an  
MUT4 primer (having the sequence as set forth in SEQ ID NO:  
20 108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
(having the sequence as set forth in SEQ ID NO: 109 in the  
Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
25 procedure as the PCR reaction No. 1.



Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and  
5 incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the  
10 dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 33<sup>rd</sup> amino  
15 acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Ala, as compared with pPT-DB1.

20 In Table 130 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 130]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 53a	33 <sup>rd</sup> position in $\beta$ -subunit	Ala	Val	gcg	gtg
No. 53b	33 <sup>rd</sup> position in $\beta$ -subunit	Ala	Met	gcg	atg

[Example 98]

Introduction of mutation for construction of modified enzyme (11)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 37<sup>th</sup> Phe of the amino acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino acid.

          Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 120 in the Sequence Listing and 50 pmol of an M13 primer M4 (having

the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which  
5 one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO:  
10 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and  
20 incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the  
25 dideoxy chain termination method using a sequencing kit and

an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 37<sup>th</sup> amino acid in the amino acid sequence of the  $\beta$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Phe, as compared with pPT-DB1.

In Table 131 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 131]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 54	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Thr	ttc	acc
No. 55	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Ala	ttc	gcc
No. 56	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Leu	ttc	ctc

No. 57	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Ile	ttc	atc
No. 58	37 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	ttc	gtc

[Example 99]

Introduction of mutation for construction of modified enzyme  
(12)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 40<sup>th</sup> Thr of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 121 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
20 used, and the reaction consisted of 25 PCR cycles, in which

one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an  
5 MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was  
10 used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the  
15 plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of  
20 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base  
25 sequence corresponding to the codon encoding the 40<sup>th</sup> amino

acid in the amino acid sequence of the  $\beta$ -subunit of  
*Pseudonocardia thermophila* JCM3095-derived nitrile hydratase  
had been changed to a base sequence corresponding to the  
codon encoding an amino acid other than Thr, as compared  
5 with pPT-DB1.

In Table 132 below, the numbers of the obtained  
transformants as well as the corresponding mutated sites,  
changes in amino acid sequence and changes in base sequence  
are given.

10 [Table 132]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 58a	40 <sup>th</sup> position in $\beta$ -subunit	Thr	Val	acg	gtg
No. 58b	40 <sup>th</sup> position in $\beta$ -subunit	Thr	Leu	acg	ctg
No. 58c	40 <sup>th</sup> position in $\beta$ -subunit	Thr	Ile	acg	att

[Example 100]

Introduction of mutation for construction of modified enzyme  
(13)

Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 41<sup>st</sup> Phe of the amino acid sequence of the  $\beta$ -subunit, which is the target of mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 122 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was



used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 41<sup>st</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Phe, as compared with pPT-DB1.

In Table 133 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 133]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 59	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Glu	ttc	gaa
No. 60	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Thr	ttc	acc
No. 61	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Ala	ttc	gcc
No. 62	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Leu	ttc	ctc
No. 63	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Ile	ttc	atc
No. 64	41 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	ttc	gtc

[Example 101]

Introduction of mutation for construction of modified enzyme

5 (14)

Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83,

mutation was performed to change the 46<sup>th</sup> Met of the amino acid sequence of the  $\beta$ -subunit, which is the target of mutation as extracted in Example 86 or 87, to another amino acid.

5        Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 123 in the Sequence Listing and 50 pmol of an M13 primer M4 (having  
10 the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15  
15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
20 (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

25        Thereafter, the same procedure as in Example 89 was

carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures  
5 were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and  
10 an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 46<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of  
15 *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Met, as compared with pPT-DB1.

In Table 134 below, the numbers of the obtained  
20 transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 134]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 65	46 <sup>th</sup> position in $\beta$ -subunit	Met	Gly	atg	ggg
No. 66	46 <sup>th</sup> position in $\beta$ -subunit	Met	Tyr	atg	tat
No. 67	46 <sup>th</sup> position in $\beta$ -subunit	Met	Leu	atg	ctg
No. 68	46 <sup>th</sup> position in $\beta$ -subunit	Met	Lys	atg	aag
No. 69	46 <sup>th</sup> position in $\beta$ -subunit	Met	Asp	atg	gat

[Example 102]

Introduction of mutation for construction of modified enzyme  
(15)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 48<sup>th</sup> Leu of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino

acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50 µl in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 124 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and

incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 48<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Leu, as compared with pPT-DB1.

In Table 135 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 135]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation

No. 70	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Gly	ctg	ggg
No. 71	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Ala	ctg	gcg
No. 72	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Val	ctg	gtg
No. 73	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Ser	ctg	tcg
No. 74	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Thr	ctg	acg
No. 75	48 <sup>th</sup> position in $\beta$ -subunit	Leu	Arg	ctg	cgg

[Example 103]

Introduction of mutation for construction of modified enzyme  
(16)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 51<sup>st</sup> Phe of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two



different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 125 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction

and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 51<sup>st</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Phe, as compared with pPT-DB1.

In Table 136 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 136]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 76	51 <sup>st</sup> position in β-subunit	Phe	Ala	ttc	gcc

No. 77	51 <sup>st</sup> position in $\beta$ -subunit	Phe	Val	ttc	gtc
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[Example 104]

Introduction of mutation for construction of modified enzyme  
(17)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 61<sup>st</sup> Ala of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 126 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
20 used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension

(72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 61<sup>st</sup> amino acid in the amino acid sequence of the β-subunit of

*Pseudonocardia thermophila* JCM3095-derived nitrile hydratase

had been changed to a base sequence corresponding to the codon encoding an amino acid other than Ala, as compared with pPT-DB1.

In Table 137 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 137]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 77a	61 <sup>st</sup> position in $\beta$ -subunit	Ala	Val	gcc	gtc
No. 77b	61 <sup>st</sup> position in $\beta$ -subunit	Ala	Leu	gcc	ctc
No. 77c	61 <sup>st</sup> position in $\beta$ -subunit	Ala	Gly	gcc	ggc

No. 77d	61 <sup>st</sup> position in $\beta$ - subunit	Ala	Ser	gcc	tcg
No. 77e	61 <sup>st</sup> position in $\beta$ - subunit	Ala	Thr	gcc	acg
No. 77f	61 <sup>st</sup> position in $\beta$ - subunit	Ala	Trp	gcc	tgg

[Example 105]

Introduction of mutation for construction of modified enzyme  
(18)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 72<sup>nd</sup> Trp of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two

different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 127 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction

and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 72<sup>nd</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Trp, as compared with pPT-DB1.

In Table 138 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 138]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 78	72 <sup>nd</sup> position	Trp	Phe	tgg	ttt



	in $\beta$ - subunit				
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[Example 106]

Introduction of mutation for construction of modified enzyme  
(19)

5       Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 112<sup>th</sup> Lys of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 128 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
20 used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension

(72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
5 (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

10        Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures  
15 were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and  
20 an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 112<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of  
25 *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase

had been changed to a base sequence corresponding to the codon encoding an amino acid other than Lys, as compared with pPT-DB1.

In Table 139 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 139]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 78a	112 <sup>th</sup> position in $\beta$ -subunit	Lys	Val	aag	gtg
No. 78b	112 <sup>th</sup> position in $\beta$ -subunit	Lys	Ile	aag	att

[Example 107]

Introduction of mutation for construction of modified enzyme (20)

Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 118<sup>th</sup> Phe of the amino

acid sequence of the  $\beta$ -subunit, which is the target of mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
5 different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 129 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence  
10 Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension  
15 (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the  
20 Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was  
25 carried out to obtain a number of transformants. Plasmids

were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 118<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Phe, as compared with pPT-DB1.

In Table 140 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 140]

Number	Mutated site	Change in amino acid sequence	Change in base sequence
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		Before mutation	After mutation	Before mutation	After mutation
No. 79	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Ala	ttc	gcc
No. 80	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Leu	ttc	ctc
No. 81	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Ile	ttc	atc
No. 82	118 <sup>th</sup> position in $\beta$ -subunit	Phe	Val	ttc	gtc

[Example 108]

Introduction of mutation for construction of modified enzyme  
(21)

5       Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 127<sup>th</sup> Leu of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of

the primer having the sequence as forth in SEQ ID NO: 130 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the

5 manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a  
10 reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the  
15 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids  
20 were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were  
25 each dissolved in a TE solution to a final concentration of

1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 127<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Leu, as compared with pPT-DB1.

In Table 141 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 141]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 83	127 <sup>th</sup> position in β-subunit	Leu	Ala	ctg	gcg



No. 84	127 <sup>th</sup> position in $\beta$ - subunit	Leu	Val	ctg	gtg
No. 85	127 <sup>th</sup> position in $\beta$ - subunit	Leu	Ser	ctg	tcg

[Example 109]

Introduction of mutation for construction of modified enzyme  
(22)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 146<sup>th</sup> Arg of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

          Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 131 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having

the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which  
5 one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO:  
10 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same  
15 procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and  
20 incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the  
25 dideoxy chain termination method using a sequencing kit and

an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 146<sup>th</sup> amino acid in the amino acid sequence of the  $\beta$ -subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Arg, as compared with pPT-DB1.

In Table 142 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 142]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 86	146 <sup>th</sup> position in $\beta$ -subunit	Arg	Gly	cgg	ggg

[Example 110]

Introduction of mutation for construction of modified enzyme  
(23)

Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
5 hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 150<sup>th</sup> Ala of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
mutation as extracted in Example 86 or 87, to another amino  
acid.

10 Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
the primer having the sequence as forth in SEQ ID NO: 132 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
15 the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
20 seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 2 minutes. For the PCR reaction No. 2, a  
reaction system of 50  $\mu$ l in total comprising 50 pmol of an  
MUT4 primer (having the sequence as set forth in SEQ ID NO:  
108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
25 (having the sequence as set forth in SEQ ID NO: 109 in the

Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

5        Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures  
10       were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and  
15       an Autosequencer 373A manufactured by ABI..

      As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 150<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of  
20       *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Ala, as compared with pPT-DB1.

      In Table 143 below, the numbers of the obtained  
25       transformants as well as the corresponding mutated sites,

changes in amino acid sequence and changes in base sequence are given.

[Table 143]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 86a	150 <sup>th</sup> position in $\beta$ -subunit	Ala	Ser	gcg	tcg
No. 86b	150 <sup>th</sup> position in $\beta$ -subunit	Ala	Asn	gcg	aat

5 [Example 111]

Introduction of mutation for construction of modified enzyme (24)

Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
10 hydratase as constructed from MT10822 in Example 83, mutation was performed to change the 160<sup>th</sup> Arg of the amino acid sequence of the  $\beta$ -subunit, which is the target of mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 133 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures

were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 160<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Arg, as compared with pPT-DB1.

In Table 144 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 144]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 87	160 <sup>th</sup>	Arg	Leu	cgg	ctg



	position in $\beta$ -subunit				
No. 88	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Trp	cgg	tgg
No. 88a	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Met	cgg	atg
No. 88b	160 <sup>th</sup> position in $\beta$ -subunit	Arg	Cys	cgg	tgt

[Example 112]

Introduction of mutation for construction of modified enzyme  
(25)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 168<sup>th</sup> Thr of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.

1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 134 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were

each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

5        As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 168<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase  
10 had been changed to a base sequence corresponding to the codon encoding an amino acid other than Thr, as compared with pPT-DB1.

      In Table 145 below, the numbers of the obtained transformants as well as the corresponding mutated sites,  
15 changes in amino acid sequence and changes in base sequence are given.

[Table 145]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 88c	168 <sup>th</sup> position in β-	Thr	acg	Glu	gag

	subunit				
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[Example 113]

Introduction of mutation for construction of modified enzyme  
(26)

5       Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 171<sup>st</sup> Lys of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of  
15 the primer having the sequence as forth in SEQ ID NO: 135 in  
the Sequence Listing and 50 pmol of an M13 primer M4 (having  
the sequence as set forth in SEQ ID NO: 107 in the Sequence  
Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
20 used, and the reaction consisted of 25 PCR cycles, in which  
one PCR cycle comprised thermal denaturation (98°C) for 15  
seconds, annealing (55°C) for 30 seconds and chain extension  
(72°C) for 2 minutes. For the PCR reaction No. 2, a

reaction system of 50  $\mu$ l in total comprising 50 pmol of an  
MUT4 primer (having the sequence as set forth in SEQ ID NO:  
108 in the Sequence Listing) and 50 pmol of an M13 primer RV  
(having the sequence as set forth in SEQ ID NO: 109 in the  
5 Sequence Listing) (for the composition of the system, the  
manufacturer's instructions for the kit were followed) was  
used, and the reaction was carried out following the same  
procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was  
10 carried out to obtain a number of transformants. Plasmids  
were prepared from the respective transformants, and the  
plasmids were subjected to addition of 30  $\mu$ g of RNase A and  
incubation at 37°C for 1 hour. Subsequently, the mixtures  
were subjected to phenol extraction/chloroform extraction  
15 and ethanol precipitation to purify the DNAs, and they were  
each dissolved in a TE solution to a final concentration of  
1.0  $\mu$ g/ $\mu$ l. The base sequences thereof were determined by the  
dideoxy chain termination method using a sequencing kit and  
an Autosequencer 373A manufactured by ABI.

20 As a result, it was found that the plasmids from the  
above-obtained transformants were such that the base  
sequence corresponding to the codon encoding the 171<sup>st</sup> amino  
acid in the amino acid sequence of the  $\beta$ -subunit of  
*Pseudonocardia thermophila* JCM3095-derived nitrile hydratase  
25 had been changed to a base sequence corresponding to the

codon encoding an amino acid other than Lys, as compared with pPT-DB1.

In Table 146 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 146]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 88d	171 <sup>st</sup> position in $\beta$ -subunit	Lys	aag	Ala	gcg

[Example 114]

10 Introduction of mutation for construction of modified enzyme (27)

Onto a plasmid: pPT-DB1 containing the ORF that codes for the *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase as constructed from MT10822 in Example 83,

15 mutation was performed to change the 176<sup>th</sup> Tyr of the amino acid sequence of the  $\beta$ -subunit, which is the target of

mutation as extracted in Example 86 or 87, to another amino acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two different types were carried out. For the PCR reaction No.

5 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 136 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the  
10 manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a  
15 reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the  
20 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids  
25 were prepared from the respective transformants, and the

plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were  
5 each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the  
10 above-obtained transformants were such that the base sequence corresponding to the codon encoding the 176<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the  
15 codon encoding an amino acid other than Tyr, as compared with pPT-DB1.

In Table 147 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence  
20 are given.

[Table 147]

Number	Mutated site	Change in amino acid sequence	Change in base sequence
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		Before mutation	After mutation	Before mutation	After mutation
No. 88e	176 <sup>th</sup> position in $\beta$ -subunit	Tyr	Ala	tac	gcc
No. 88f	176 <sup>th</sup> position in $\beta$ -subunit	Tyr	Met	tac	atg
No. 88g	176 <sup>th</sup> position in $\beta$ -subunit	Tyr	Cys	tac	tgc
No. 88h	176 <sup>th</sup> position in $\beta$ -subunit	Tyr	Thr	tac	acc

[Example 115]

Introduction of mutation for construction of modified enzyme  
(28)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 186<sup>th</sup> Leu of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of

the primer having the sequence as forth in SEQ ID NO: 137 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the

5 manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a  
10 reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the  
15 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids  
20 were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were  
25 each dissolved in a TE solution to a final concentration of

1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 186<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Leu, as compared with pPT-DB1.

In Table 148 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 148]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 89	186 <sup>th</sup> position in β-subunit	Leu	Glu	ctg	gag
No. 90	186 <sup>th</sup> position in β-subunit	Leu	Asp	ctg	gat

No. 91	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Lys	ctg	aag
No. 92	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Arg	ctg	cgg
No. 93	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Asn	ctg	aac
No. 94	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Ser	ctg	tcg
No. 95	186 <sup>th</sup> position in $\beta$ -subunit	Leu	Gly	ctg	ggg

[Example 116]

Introduction of mutation for construction of modified enzyme  
(29)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 217<sup>th</sup> Asp of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two  
different types were carried out. For the PCR reaction No.  
1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of

the primer having the sequence as forth in SEQ ID NO: 138 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the  
5 manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a  
10 reaction system of 50 µl in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the  
15 manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids  
20 were prepared from the respective transformants, and the plasmids were subjected to addition of 30 µg of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction and ethanol precipitation to purify the DNAs, and they were  
25 each dissolved in a TE solution to a final concentration of

1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 217<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Asp, as compared with pPT-DB1.

In Table 149 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 149]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No. 96	217 <sup>th</sup> position in β-subunit	Asp	Gly	gac	ggc
No. 96a	217 <sup>th</sup> position in β-subunit	Asp	Val	gac	gtc

No. 96b	217 <sup>th</sup> position in $\beta$ -subunit	Asp	Leu	gac	ctc
No. 96c	217 <sup>th</sup> position in $\beta$ -subunit	Asp	Met	gac	atg
No. 96d	217 <sup>th</sup> position in $\beta$ -subunit	Asp	Cys	gac	tgt
No. 96e	217 <sup>th</sup> position in $\beta$ -subunit	Asp	Ser	gac	agc
No. 96f	217 <sup>th</sup> position in $\beta$ -subunit	Asp	Thr	gac	acc
No. 96g	217 <sup>th</sup> position in $\beta$ -subunit	Asp	His	gac	cac

[Example 117]

Introduction of mutation for construction of modified enzyme  
(30)

5           Onto a plasmid: pPT-DB1 containing the ORF that codes  
for the *Pseudonocardia thermophila* JCM3095-derived nitrile  
hydratase as constructed from MT10822 in Example 83,  
mutation was performed to change the 218<sup>th</sup> Cys of the amino  
acid sequence of the  $\beta$ -subunit, which is the target of  
10 mutation as extracted in Example 86 or 87, to another amino  
acid.

Using 10 ng of pPT-DB1 as the template, PCRs of two

different types were carried out. For the PCR reaction No. 1, a reaction system of 50  $\mu$ l in total comprising 50 pmol of the primer having the sequence as forth in SEQ ID NO: 139 in the Sequence Listing and 50 pmol of an M13 primer M4 (having the sequence as set forth in SEQ ID NO: 107 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction consisted of 25 PCR cycles, in which one PCR cycle comprised thermal denaturation (98°C) for 15 seconds, annealing (55°C) for 30 seconds and chain extension (72°C) for 2 minutes. For the PCR reaction No. 2, a reaction system of 50  $\mu$ l in total comprising 50 pmol of an MUT4 primer (having the sequence as set forth in SEQ ID NO: 108 in the Sequence Listing) and 50 pmol of an M13 primer RV (having the sequence as set forth in SEQ ID NO: 109 in the Sequence Listing) (for the composition of the system, the manufacturer's instructions for the kit were followed) was used, and the reaction was carried out following the same procedure as the PCR reaction No. 1.

Thereafter, the same procedure as in Example 89 was carried out to obtain a number of transformants. Plasmids were prepared from the respective transformants, and the plasmids were subjected to addition of 30  $\mu$ g of RNase A and incubation at 37°C for 1 hour. Subsequently, the mixtures were subjected to phenol extraction/chloroform extraction



and ethanol precipitation to purify the DNAs, and they were each dissolved in a TE solution to a final concentration of 1.0 µg/µl. The base sequences thereof were determined by the dideoxy chain termination method using a sequencing kit and an Autosequencer 373A manufactured by ABI.

As a result, it was found that the plasmids from the above-obtained transformants were such that the base sequence corresponding to the codon encoding the 218<sup>th</sup> amino acid in the amino acid sequence of the β-subunit of *Pseudonocardia thermophila* JCM3095-derived nitrile hydratase had been changed to a base sequence corresponding to the codon encoding an amino acid other than Cys, as compared with pPT-DB1.

In Table 150 below, the numbers of the obtained transformants as well as the corresponding mutated sites, changes in amino acid sequence and changes in base sequence are given.

[Table 150]

Number	Mutated site	Change in amino acid sequence		Change in base sequence	
		Before mutation	After mutation	Before mutation	After mutation
No.	218 <sup>th</sup> position	Cys	Met	tgc	atg

96h	in $\beta$ -subunit				
No.	218 <sup>th</sup> position	Cys	Ser	tgc	tcc
96i	in $\beta$ -subunit				

[Example 118]

Comparison of character between nitrile hydratase before modification and modified enzyme after modification (1)

5        In a 500 ml Erlenmeyer flask with baffles, 5 sets of a liquid LB medium of 100 ml each containing 40  $\mu$ g/ml of ferric sulfate·heptahydrate and 10  $\mu$ g/ml of cobalt chloride·hexahydrate were prepared and sterilized by autoclaving at 121°C for 20 minutes. To each medium,  
10        ampicillin was added to a final concentration of 100  $\mu$ g/ml.

Five types of transformants such as transformant No. 200 obtained in Example 84 and transformants No. 201 to 204 obtained in Example 88 were inoculated onto the above media such that one platinum loop of each of the transformant was  
15        inoculated on each of the five media. The cells were incubated therein at 37°C for about 20 hours with stirring at 130 rpm. Each of the transformants were separated from the resulting culture by centrifugation (5,000 G  $\times$  15 minutes). Subsequently, the separated transformants were  
20        resuspended in 50 ml of physiological saline, and then subjected to another centrifugation (5,000 G  $\times$  15 minutes),

thereby each transformant being separated.

0.1 g of each of the transformants were suspended in 20 ml of an aqueous solution (pH 7.0) of 50mM potassium phosphate, and then the suspensions were divided into 10 ml  
5 × 2 sets. Thus, two suspensions per each transformant, that is, 10 suspensions in total, were prepared. To one suspension of each transformant, 1 ml of acrylonitrile was added, and to the other suspension, methacrylonitrile was added. The suspensions were gently stirred at 30°C for 10  
10 minutes to react.

After completion of the reaction, an analysis of the reaction mixtures was carried out with HPLC, and it was found that each mixture contained a nitrile compound (acrylonitrile or methacrylonitrile), which was unreacted  
15 substrate, and an amide compound (acrylamide or methacrylamide), which was the corresponding product from the reaction. Further, the presence of a corresponding organic acid (acrylic acid or methacrylic acid) was not detected.

20 Comparison was made on the molar ratios for the respective transformants between the acrylamide which was produced from the reaction employing acrylonitrile as the substrate, and the methacrylamide which was produced from the reaction employing methacrylonitrile as the substrate.  
25 Differences as indicated in Table 151 below were observed.

When a comparison is made in terms of acrylonitrile versus methacrylonitrile, from the viewpoint that methacrylonitrile is a bulkier nitrile compound, the results show that the obtained modified enzymes had increased facility in

5 hydration of a bulkier substrate.

[Table 151]

Number	48 <sup>th</sup> amino acid in $\beta$ -subunit (base sequence)	Ratio of hydration activation (relative ratio)
		[case with methacrylonitrile as substrate] $\div$ [case with acrylonitrile as substrate]
No. 200	Trp (tgg)	0.41 (100%)
No. 201	Tyr (tat)	0.87 (211%)
No. 202	Val (gtg)	0.79 (192%)
No. 203	Ala (gcg)	0.67 (163%)
No. 204	Gly (ggg)	0.82 (198%)

[Example 119]

Comparison of character between nitrile hydratase before

modification and modified enzyme after modification (2)

In a 500 ml Erlenmeyer flask with baffles, 57 sets of a liquid LB medium of 100 ml each containing 40 µg/ml of ferric sulfate·heptahydrate and 10 µg/ml of cobalt

5 chloride·hexahydrate were prepared and sterilized by autoclaving at 121°C for 20 minutes. To each medium, ampicillin was added to a final concentration of 100 µg/ml.

Fifty-seven types of transformants such as a transformant No. 0 obtained by transforming HB101 with pPT-  
10 DB1 and the following transformants obtained in Examples 89 to 117: No. 40, No. 40e, No. 40f, No. 42, No. 42a, No. 43, No. 44, No. 45, No. 46, No. 47, No. 48, No. 49, No. 50, No. 51, No. 52, No. 54, No. 55, No. 56, No. 57, No. 58, No. 59, No. 60, No. 61, No. 62, No. 63, No. 64, No. 65, No. 66, No.  
15 67, No. 68, No. 69, No. 70, No. 71, No. 72, No. 73, No. 74, No. 75, No. 76, No. 77, No. 78, No. 79, No. 80, No. 81, No. 82, No. 83, No. 84, No. 85, No. 87, No. 88, No. 89, No. 90, No. 91, No. 92, No. 93, No. 94 and No. 95, were inoculated onto the above media such that one platinum loop of each of  
20 the transformant was inoculated on each of the 56 media.

The cells were incubated therein at 37°C for about 20 hours with stirring at 130 rpm. Each of the transformants was separated from the resulting culture by centrifugation (5,000 G × 15 minutes). Subsequently, the separated

25 transformants were resuspended in 50 ml of physiological

saline, and then subjected to another centrifugation (5,000 G × 15 minutes), thereby each transformant being separated.

0.1 g of each of the transformants were suspended in 20 ml of an aqueous solution (pH 7.0) of 50mM potassium phosphate, and then the suspensions were divided into 10 ml × 2 sets. Thus, two suspensions per each transformant, that is, 10 suspensions in total, were prepared. To one suspension of each transformant, 1 ml of acrylonitrile was added, and to the other suspension, methacrylonitrile was added. The suspensions were gently stirred at 20°C for 10 minutes to react.

After completion of the reaction, an analysis of the reaction mixtures was carried out with HPLC, and it was found that each mixture contained a nitrile compound (acrylonitrile or methacrylonitrile), which was unreacted substrate, and an amide compound (acrylamide or methacrylamide), which was the corresponding product from the reaction. Further, the presence of a corresponding organic acid (acrylic acid or methacrylic acid) was not detected.

Comparison was made on the molar ratios for the respective transformants between the acrylamide which was produced from the reaction employing acrylonitrile as the substrate, and the methacrylamide which was produced from the reaction employing methacrylonitrile as the substrate.

Thus, such diversity as indicated in Table 152, Table 153 and Table 154 could be observed. When acrylonitrile and methacrylonitrile are compared for their bulkiness, methacrylonitrile is the bulkier nitrile compound. These results show that modified enzymes with changed substrate specificity was obtained, as compared with nitrile hydratase before modification.

[Table 152]

Number	Ratio of hydration activation (relative ratio)	Ratio of hydration activation (relative ratio)
	[case with methacrylonitrile as substrate] ÷ [case with acrylonitrile as substrate]	[case with acrylonitrile as substrate] ÷ [case with methacrylonitrile as substrate]
No. 0	0.189 (100%)	5.29 (100%)
No. 40	0.219 (116%)	4.55 (86%)
No. 40e	0.211 (112%)	4.71 (89%)
No. 40f	0.206 (109%)	4.87 (92%)
No. 42	0.200 (106%)	4.97 (94%)
No. 42a	0.202 (107%)	4.92 (93%)
No. 43	0.185 (98%)	5.40 (102%)

No. 44	0.185 (98%)	5.40 (102%)
No. 45	0.187 (99%)	5.34 (101%)
No. 46	0.185 (98%)	5.40 (102%)
No. 47	0.181 (96%)	5.50 (104%)
No. 48	0.187 (99%)	5.34 (101%)
No. 49	0.208 (110%)	4.81 (91%)
No. 50	0.198 (105%)	5.03 (95%)
No. 51	0.187 (99%)	5.34 (101%)
No. 52	0.202 (107%)	4.92 (93%)
No. 54	0.153 (81%)	6.51 (123%)
No. 55	0.159 (84%)	6.30 (119%)
No. 56	0.204 (108%)	4.92 (93%)
No. 57	0.168 (89%)	5.92 (112%)

[Table 153]

Number	Ratio of hydration activation (relative ratio)	Ratio of hydration activation (relative ratio)
	<div> <div>[case with methacrylonitrile as substrate]</div> <div>÷</div> <div>[case with acrylonitrile as substrate]</div> </div>	<div> <div>[case with acrylonitrile as substrate]</div> <div>÷</div> <div>[case with methacrylonitrile as substrate]</div> </div>



No. 58	0.159 (84%)	6.30 (119%)
No. 59	0.183 (97%)	5.45 (103%)
No. 60	0.160 (85%)	6.24 (118%)
No. 61	0.195 (103%)	5.13 (97%)
No. 62	0.210 (111%)	4.76 (90%)
No. 63	0.198 (105%)	5.03 (95%)
No. 64	0.155 (82%)	6.45 (122%)
No. 65	0.206 (109%)	4.87 (92%)
No. 66	0.180 (95%)	5.55 (105%)
No. 67	0.172 (91%)	5.82 (110%)
No. 68	0.399 (211%)	2.49 (47%)
No. 69	0.259 (137%)	3.86 (73%)
No. 70	0.212 (112%)	4.71 (89%)
No. 71	0.223 (118%)	4.50 (85%)
No. 72	0.206 (109%)	4.87 (92%)
No. 73	0.229 (121%)	4.39 (83%)
No. 74	0.233 (123%)	4.28 (81%)
No. 75	0.204 (108%)	4.92 (93%)
No. 76	0.191 (101%)	5.24 (99%)
No. 77	0.271 (142%)	3.70 (70%)

[Table 154]

Number	Ratio of hydration activation (relative ratio)	Ratio of hydration activation (relative ratio)
	[case with methacrylonitrile as substrate] ÷ [case with acrylonitrile as substrate]	[case with acrylonitrile as substrate] ÷ [case with methacrylonitrile as substrate]
No. 78	0.268 (137%)	3.86 (73%)
No. 79	0.170 (90%)	5.87 (111%)
No. 80	0.183 (97%)	5.45 (103%)
No. 81	0.180 (95%)	5.56 (105%)
No. 82	0.164 (87%)	6.08 (115%)
No. 83	0.174 (92%)	5.77 (109%)
No. 84	0.208 (110%)	4.81 (91%)
No. 85	0.164 (87%)	6.08 (115%)
No. 87	0.176 (93%)	5.71 (108%)
No. 88	0.166 (88%)	6.03 (114%)
No. 89	0.191 (101%)	5.24 (99%)
No. 90	0.197 (104%)	5.08 (96%)
No. 91	0.187 (99%)	5.34 (101%)
No. 92	0.185 (98%)	5.40 (102%)
No. 93	0.187 (99%)	5.34 (101%)

No. 94	0.193 (102%)	5.18 (98%)
No. 95	0.187 (99%)	5.34 (101%)

#### INDUSTRIAL APPLICABILITY

The present invention is useful in the art of material  
5 production using biological catalyst. An example thereof  
may include the case of material production by conversion of  
a nitrile compound to a corresponding amide compound by  
means of hydration using an enzyme called nitrile hydratase  
or an organism expressing the enzyme activity as the  
10 biological catalyst.